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# Interrelationship between in vivo Fluorescence of Phytoplankton and Light Beam Transmission with Reference to Fluorescence Yield[1]

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Vandevelde, T., L. Legendre, S. Demers, and J.-C. Therriault. 1988. Interrelationship between in vivo fluorescence of phytoplankton and light beam transmission with reference to fluorescence yield. Can. J. Fish. Aquat. Sci. 45: 1508–1513.

In vivo fluorescence and light beam transmission were simultaneously recorded during two cruises in the St. Lawrence estuary. Marked differences in the relationship between light beam attenuation (c(660)) and in vivo fluorescence (IVF) were observed (1) along a short transect and (2) between surface (<5 m) and underlying waters (5–15 m) for a series of vertical profiles at an anchor station. In both cases, the observed differences were related to changes in fluorescence yield. At the anchor station, however, several other factors could also have affected the c(660)–IVF relationship. Below 5 m, tight linear relationships between c(660) and IVF indicate that no major change took place in either fluorescence yield or the concentration of chlorophyll per cell. In such a case, the two measurements provide consistent estimates of phytoplankton biomass.

La fluorescence in vivo et le coefficient d'atténuation d'un faisceau lumineux (c(660)) furent mesurés simultanément au cours de deux missions dans l'estuaire du Saint-Laurent. Des variations importantes dans la relation entre l'atténuation à 660 nm (c(660)) et la fluorescence in vivo (IVF) ont été observées 1) le long d'une radiale et 2) entre les eaux de surface (< 5 m) et les eaux sous-jacentes (5–15 m) pour une série de profils verticaux en station fixe. Dans les deux cas, les variations observées sont liées à des changements du rendement en fluorescence. À la station fixe, toutefois, plusieurs autres facteurs ont pu affecter la relation c(660)–IVF. La liaison étroite, observée en dessous de 5 m entre c(660) et IVF indique qu'aucun changement important ne s'est produit dans le rendement en fluorescence ou la concentration de chlorophylle par cellule. Dans un tel cas, les deux mesures fournissent une estimation valide de la biomasse phytoplanctonique.

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Since Lorenzen (1966), in vivo fluorescence (IVF) has been used to estimate in situ phytoplankton concentrations in the aquatic environment. Caution should be exercised, however, when using IVF as an index of phytoplankton biomass (Loftus and Seliger 1975; Lorenzen and Jeffrey 1980; Prézelin 1981; Cullen 1982; Falkowski and Kiefer 1985). Several studies have indicated a large range of variation in the fluorescence yield (IVF per unit chlorophyll *a*), both in time and space (Blasco 1973; Kiefer 1973; Loftus and Seliger 1975; Heaney 1978; Vincent 1979; Harris 1980). Also, fluorescence yield is known to vary with different species (Alpine and Cloern 1985) and different environments (Kiefer 1973; Harris 1980), and at times of high light intensity, it has been observed to vary as a consequence of the susceptibility of fluorescence to photoinhibition (Kiefer 1973; Heaney 1978; Günther 1986). In addition, several factors can greatly influence the amount of chlorophyll per cell (see Richardson et al. 1983) and consequently in vivo fluorescence.

In situ distributions of phytoplankton have also been estimated from light beam transmission (LBT) (Baker and Baker 1976). This optical measurement is commonly used as an index for the concentration of suspended particulate matter (Bartz et al. 1978; Pak 1986; Spinrad 1986; Siegel and Dickey 1987). Baker and Baker (1976) have shown that a good correlation could exist between LBT and the number of phytoplankton cells, so that LBT can be used, in specific cases, to study the distribution of phytoplankton. Despite the numerous sources of variation that could independently affect the values of IVF (see above) or LBT (e.g. size and shape or refractive index of particles), the two measurements are often tightly related. For example, Kiefer and Austin (1974) reported good correlations between IVF and LBT for various waters in the Gulf of California.

In the present study, simultaneous measurements of IVF and LBT were conducted in the St. Lawrence estuary. LBT data were systematically compared with IVF to (1) assess how far the two measurements can provide consistent estimates of phytoplankton biomass in a physically variable environment and (2) specify under which conditions qualitative or quantitative informations on physiological characteristics of phytoplankton

[1]Contribution to the programs of GIROQ (Groupe interuniversitaire de recherches océanographiques du Québec) and of the Maurice Lamontagne Institute (Department of Fisheries and Oceans).

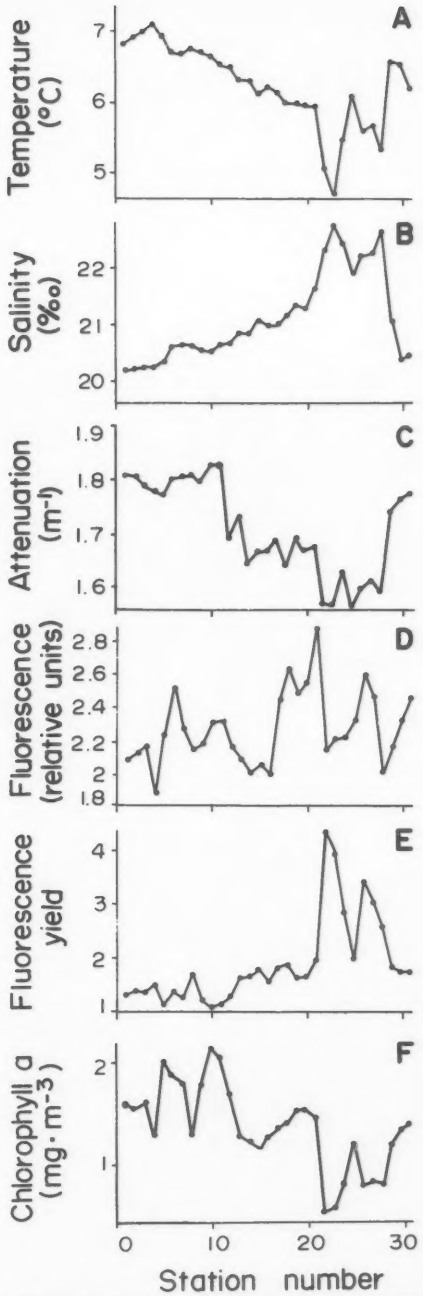


FIG. 1. Transect (6 km): (A) temperature, (B) salinity, (C) light beam attenuation c(660), (D) IVF, (E) fluorescence yield of chlorophyll *a* (fluorescence/Chl *a*), and (F) extracted chlorophyll *a*.

(e.g. fluorescence yield of chlorophyll, cellular chlorophyll content) can be derived from such a comparison.

## Materials and Methods

LBT was measured at 660 nm using a Sea Tech 25-cm beam transmissometer. Optical design of the instrument features a collimated transmitter (light-emitting diode) with a beam divergence of less than 5 mrad and a receiver with an acceptance angle of less than 18 mrad in water (Bartz et al. 1978). The coefficient of beam attenuation ($m^{-1}$) was computed from measured transmittance ($T$):

$$\text{Beam attenuation coefficient } (c) = -(\ln T)/0.25 \text{ m}.$$

On 2 June 1983, a short transect was sampled at midday along the north shore of the lower St. Lawrence estuary (from 48°51.40′N, 68°43.29′W to 48°51.28′N, 68°46.29′W; 1-h transect). Water pumped from 5 m (~10% of surface light) was continuously monitored on deck for IVF (Turner Designs fluorometer), temperature, and salinity (Guildline 8770 CTD adapted for measurements on pumped water). Every 2 min, water samples were collected at the outlet of the fluorometer and filtered through Whatman GF/C glass fiber filters. After a 24-h extraction in 90% acetone, the concentration of chlorophyll *a* was determined using the fluorometric method of Yentsch and Menzel (1963) as modified by Holm-Hansen et al. (1965). Continuous series of temperature, salinity, IVF, and LBT were numerically filtered (90-s moving averages), and discrete values were sampled from the filtered series at 2-min intervals.

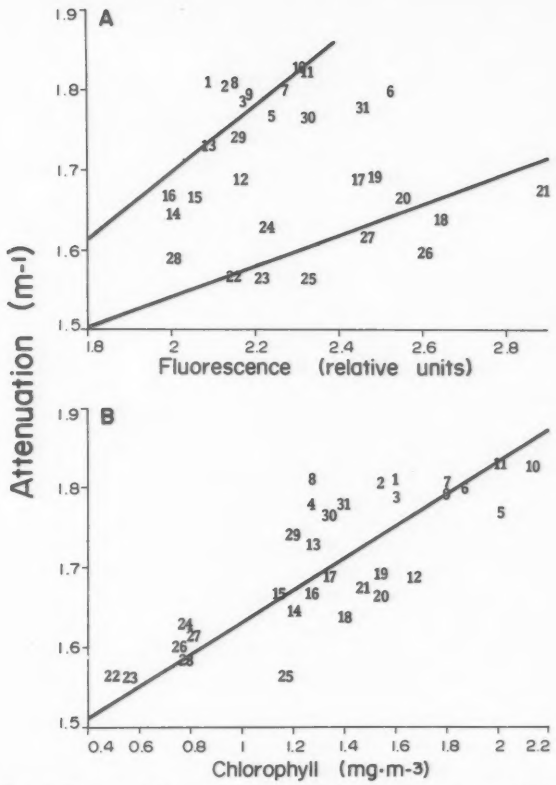


FIG. 2. Transect (6 km): coefficient of light beam attenuation (c(660)) as a function of (A) IVF and (B) extracted chlorophyll *a*. Equations of model II linear regressions (reduced major axis; Ricker 1973; Sokal and Rohlf 1981) in Fig. 2A are c(660) = 0.41IVF + 0.87 (stations 1–16) and c(660) = 0.14IVF + 1.23 (stations 20–28).

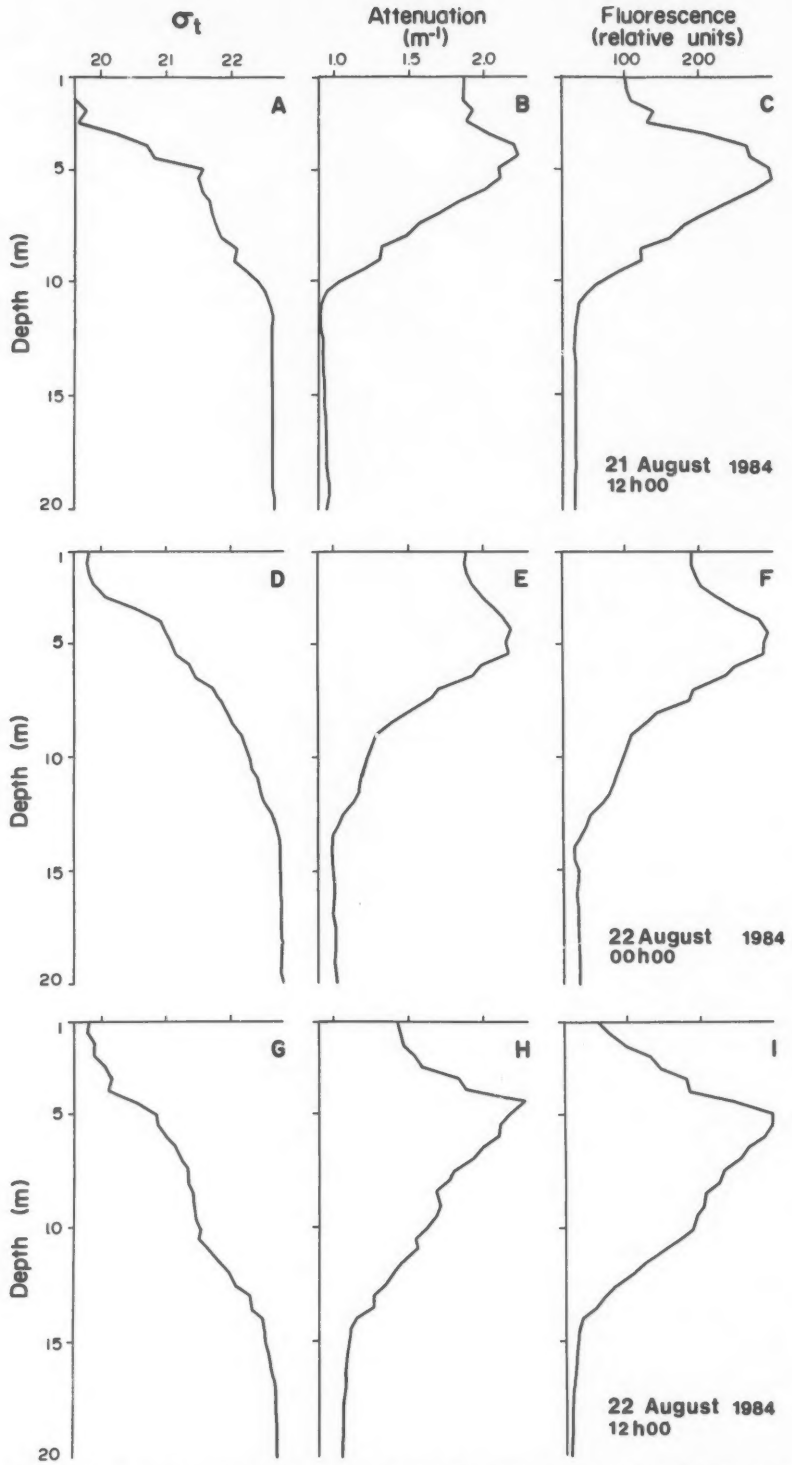


FIG. 3. Typical vertical profiles of $\sigma_t$, coefficient of light beam attenuation c(660), and IVF during the 96-h anchor station.

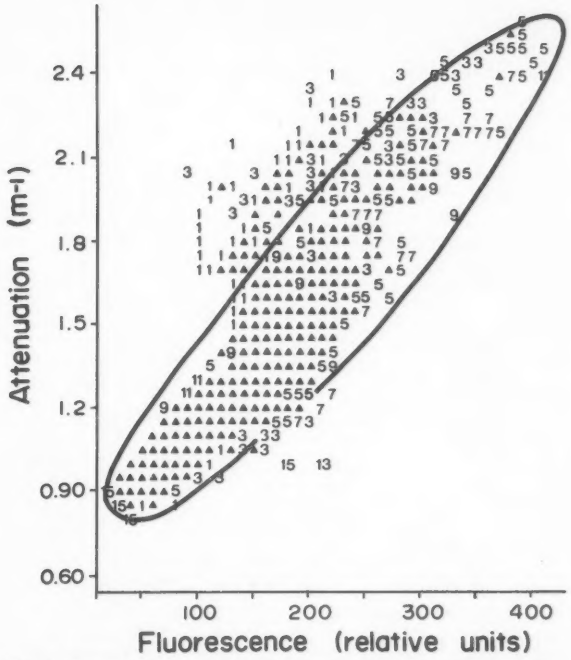


FIG. 4. Coefficient of light beam attenuation c(660) versus IVF fluorescence during the 96-h anchor station. Discrete data ($n$ = 2312) from eight depths (1, 3, 5, 7, 9, 11, 13, and 15 m) as measured every 20 min. Points represent superimposed observations, and the envelope contains observations >5 m.

In 1984, sampling was conducted at an anchor station in the estuary (48°51.7′N, 67°36.15′W; 62 m depth) from 20 to 24 August. An automatic yo-yo profiler interfaced with a computer was used to record more than 1000 vertical profiles of temperature, salinity, IVF, and LBT. IVF was measured on deck on water continuously pumped from the yo-yo profiler. Measurements were recorded on the computer at intervals of 0.5 m after shifting back the fluorescence data by a time lag corresponding to the estimated time of water circulation in an opaque hose between the pump intake and the fluorometer. The flow, continuously monitored with an electronic flowmeter at the outlet of the fluorometer, was constant for the whole sampling period. The residence time in the hose was short enough (<50 s) to prevent significant variations of fluorescence yield prior to measurement (Sakshaug et al. 1987). Data were analyzed using scatter diagrams of LBT (beam attenuation coefficient) versus IVF (see Fig. 2A, 4, 5) by reference to Kiefer and Austin (1974). These authors have shown that variations in LBT are associated with changes in the concentration of phytoplankton (fluorescent material) and other particles, the latter comprising a fraction that covaries with the fluorescent material and another (minerogenic fraction) that does not. According to this model, as long as optical characteristics of the cells and the medium are the same, the relationship between LBT and IVF should remain constant and linear. On the other hand, variations in these characteristics should change the LBT–IVF relationship. For example, a variation in fluorescence yield of the cells should cause a change in the slope of the relationship. Similarly, a variation in the minerogenic fraction should change the $y$-intercept. Scatter diagrams of LBT versus IVF can thus be used to explore our initial questions.

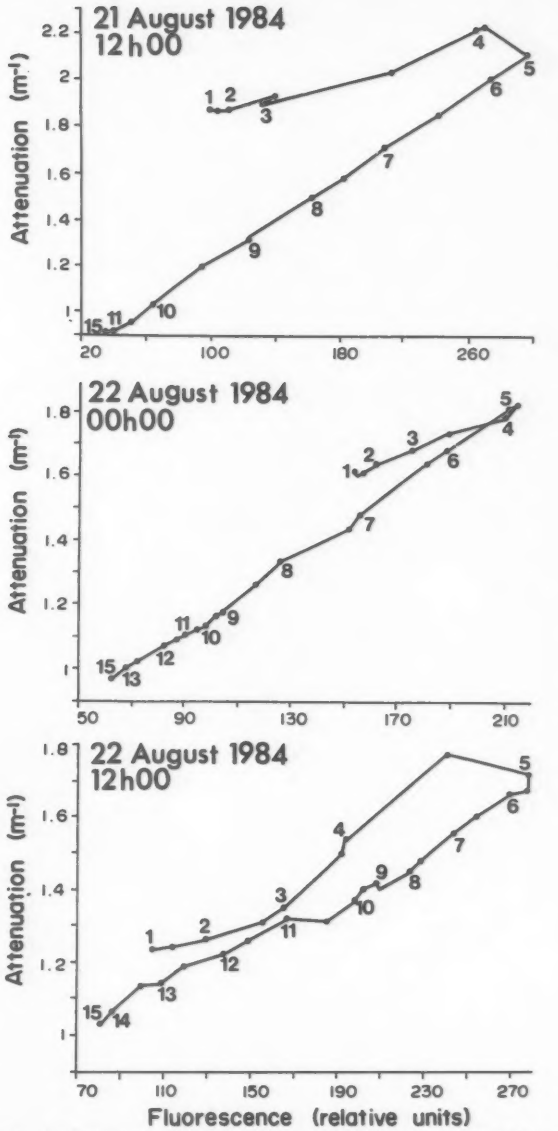


FIG. 5. Coefficient of light beam attenuation c(660) versus IVF for three vertical profiles during the 96-h anchor station (same profiles as in Fig. 3).

## Results and Discussion

### Transect

Figure 1 shows the data series along the 1-h transect. Upwelled waters were encountered at the end of the transect, as clearly shown by rapid changes in temperature and salinity (Fig. 1A, 1B) observed after the 20th station. In these waters, fluorescence yield of chlorophyll *a* was high (IVF/Chl *a*, Fig. 1E). The scatter diagram (Fig. 2A) shows that there is no overall relationship between attenuation (c(660)) and IVF for the whole transect. There were in fact two distinct clouds of points. The first one consists of data collected in the upwelled

waters (Fig. 1: stations 20–28), where fluorescence yield per unit of chlorophyll *a* was high (Fig. 1E). The second one corresponds to stations located outside the upwelled waters, where the cells were characterized by lower fluorescence yield. Regressions between c(660) on IVF (Fig. 2A) show a significant ($P \leq 0.05$) linear relationship for each cloud of points. The two lines have different slopes ($P \leq 0.05$), but there were no significant differences in intercepts (i.e. null fluorescence would have yielded an attenuation of about 1 $m^{-1}$ in all cases), which suggests a relatively constant background of nonfluorescent particles along the transect. Figures 1E and 2A indicate that changes evidenced in the attenuation–fluorescence relationship (Fig. 2A) were mainly caused, in this case, by changes in fluorescence yield. This is confirmed by the migration of all the stations toward a single regression line ($r = 0.79$, $P \leq 0.01$), when extracted chlorophyll *a* is used instead of IVF (Fig. 2B). If changes had rather been caused by varying amounts of chlorophyll per cell, similar changes in the attenuation–fluorescence relationship would have been observed but the attenuation–chlorophyll relationship would have also been affected which was not the case here (Fig. 2B). On the other hand, variations in the background of nonfluorescent particles without changes in the characteristics of the phytoplankton cells would have resulted in changing the intercepts but not the slopes. These results show that an increase in fluorescence yield (~3 times) results in a proportional decrease in the slope of the linear relationship between c(660) and IVF (from 0.41 to 0.14). In the present case, slopes of regressions line in Fig. 2A would provide reliable estimates of fluorescence yield of the cells.

### Anchor Station

In general, a good correspondence was observed between vertical profiles of c(660) and IVF during 96 h of sampling at the anchor station (Fig. 3). The scatter diagram of c(660) versus IVF (Fig. 4) shows a general linear relationship between the two variables ($n = 2312$, $r = 0.87$, $P \leq 0.01$). However, some points from surface waters (1 and 3 m) fall outside the envelope that contains the observations >5 m. Examination of scatter diagrams plotted for typical profiles (Fig. 5) confirms that the relationship between c(660) and IVF was not the same for surface (<5 m) and deeper waters and shows that the largest differences were observed near midday. One possibility to explain this difference would therefore be photoinhibition of fluorescence during periods of high irradiance, especially that the surface mixed layer was <5 m. For observations at 1 m, removing the 44 data points corresponding to PAR > 300 $\mu Ein \cdot m^{-2} \cdot s^{-1}$ increases the correlation between c(660) and IVF from 0.65 ($n = 289$, $P < 0.01$) to 0.84 ($n = 245$, $P < 0.01$) which would support the hypothesis of photoinhibition. However, when model II regressions of c(660) on IVF are computed for both the 245 low-light observations (<300 $\mu Ein \cdot m^{-2} \cdot s^{-1}$) at 1 m and those from deeper waters (7–15 m), significant differences ($P < 0.01$) are found between the two data sets in the slopes and the intercepts. This shows that photoinhibition of fluorescence explains only part of the differences observed between surface and deeper waters (Fig. 5).

Mechanisms other than changes in the fluorescence yield must be invoked to explain differences observed with depth in the LBT–IVF relationship between the surface and underlying waters. Lieberman et al. (1984) have ascribed vertical variations in the LBT–IVF relationship to changes with depth in the proportion of nonfluorescent versus fluorescent particles. In the present case, differences observed between surface and underlying waters could thus be in part attributed to changes in the proportion of nonfluorescent particulate matter. According to Kiefer (1984), such vertical variations could also be explained by changes in the amount of chlorophyll per cell associated with photoadaptation. For example, an increase in the amount of chlorophyll per cell (shade adaptation) can lead to higher fluorescence for the same number of cells (same attenuation). The above mechanisms would primarily affect IVF. Other mechanisms could mainly affect LBT; for example, changes in the size spectrum of particles with depth (Kitchen et al. 1982; Campbell and Spinrad 1987).

Changes in the relationship between c(660) and IVF, as observed along the transect (Fig. 2A) or at the anchor station between surface and deeper water (Fig. 4), indicate potential problems in estimating phytoplankton biomass from either IVF or c(660)). For the transect, it was possible to identify a single cause for the changes that occurred in the c(660)–IVF relationship (i.e. fluorescence yield of chlorophyll *a*), which was not the case for surface waters at the anchor station. A first conclusion of the present study is that it is often not possible to identify which factors are responsible for changes in c(660)–IVF relationships, in the absence of additional measurements (e.g. fluorescence yield, particle size spectra, scattering coefficient, etc.). A second conclusion is that interesting biological information can be extracted when c(660) and IVF are linearly related. As an example, the tight relationship between fluorescence and attenuation below 5 m at the anchor station indicates that no major changes took place between 5 and 15 m in either the fluorescence yield or the concentration of chlorophyll per cell. In such a case, the two measurements provide consistent estimates of phytoplankton biomass.

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# Uncoupling of Somatic and Otolith Growth Rates in Arctic Char (*Salvelinus alpinus*) as an Effect of Differences in Temperature Response

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Experiments were conducted to study the effect of temperature, fish size, and somatic growth rate on mean daily otolith growth rate in Arctic char (*Salvelinus alpinus*) fry fed maximum rations. Long-term experiments at two different temperatures (8 and 13°C) showed exponential relationships between otolith weight and fish wet weight, with the exponent of wet weight for the higher temperature being significantly higher than for the lower temperature. Increasing exponents at each sampling date indicated a difference in growth rates of the char and its otoliths. Short-term experiments showed that specific growth rate of the fry at six different temperatures was that of an optimum curve whereas the otolith growth rate continued to increase with increasing temperatures. A clear uncoupling between otolith growth rate and fish growth rate was demonstrated whereas temperature and fish wet weight modeled otolith growth rate reasonably well. It was found that otolith growth rate expressed as daily increase in weight gave a model with better fit and higher generality than growth rate along various radii. Several examples of uncoupling between fish growth rate and otolith growth rate in the literature were reexamined, and an explanation of otolith growth rate in terms of a metabolic expression was suggested.

On a procédé à des expériences pour déterminer l'effet de la température, de la taille des poissons et du taux de croissance somatique sur le taux de croissance moyen quotidien des otolithes chez des alevins d'omble arctique (*Salvelinus alpinus*) auxquels on a donné des rations alimentaires maximales. Les résultats d'expériences à long terme réalisées à deux températures différentes (8 et 15°C) ont révélé des relations exponentielles entre le poids d'un otolithe et le poids humide du poisson; l'exposant du poids humide pour la température élevée était significativement plus grand que celui correspondant à la basse température. L'augmentation des exposants à chaque date d'échantillonnage a montré qu'il y avait une différence dans les taux de croissance de l'omble et de ses otolithes. Les résultats d'expériences à court terme ont révélé que le taux de croissance caractéristique des alevins à six températures différentes suivait une courbe optimale, tandis que le taux de croissance des otolithes a continué à augmenter avec une augmentation de la température. On a démontré une dissociation nette entre le taux de croissance des otolithes et le taux de croissance des poissons, tandis que la température et le poids humide des poissons réglaient assez bien le taux de croissance des otolithes. On a établi que le taux de croissance des otolithes correspondant à une augmentation quotidienne de poids donnait un modèle mieux ajusté et plus général que le taux de croissance le long de divers rayons. On a réexaminé plusieurs exemples publiés de dissociation entre le taux de croissance des poissons et le taux de croissance des otolithes et on a donné une explication du taux de croissance des otolithes en termes d'expression du métabolisme.

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The literature on otolith microstructures has steadily increased since Pannella (1971) found that teleosts deposit daily increments in the otoliths. Age determination with the greatest precision was the obvious first application (Pannella 1971, 1974) and has thus far been a major objective in many studies (Gjösäter et al. 1984). The more involved analyses of back-calculating growth have been suggested (Struhsaker and Uchiyama 1976). Although the calculation of a daily growth rate in fish would offer a welcome step forward in fisheries biology, studies delving into the detailed fundamentals of such methods are few (Campana and Neilson 1985).

The method of back-calculation is based on a relationship between fish size and otolith size that has one unique solution for fish size corresponding to a particular otolith size. Little evidence has been gathered to support a link between fish growth rate and otolith incremental width (Neilson and Geen 1982, 1985; Wilson and Larkin 1982; Volk et al. 1984) and none with the necessary resolution to determine the relationship on a daily basis (Campana and Neilson 1985). Indeed, several studies have presented examples of complex variations in the relationship between fish size and otolith size (Brothers 1981; Wilson and Larkin 1982; Marshall and Parker 1982; Campana 1983; Rice et al. 1985).

Experiments involving simultaneous studies of fish and otolith growth have shown a positive correlation between fish growth rate and otolith increment width (Volk et al. 1984; Neilson and Geen 1985). However, the former study and others as well have shown a continuous growth of otoliths during star-

vation or negative growth (Brothers 1981; Marshall and Parker 1982; Campana 1983; Volk et al. 1984). Apparently due to the many discrepancies from a dependent coupling between fish and otolith growth, the hypothesis of a functional relationship has been modified ad hoc to say "otolith growth represents a running average of fish growth rates, with the run inversely proportional to metabolic activity" (Campana and Neilson 1985).

In a study on otolith growth rate in salmonid yolk sac fry, Mosegaard and Titus (1987) found a clear uncoupling between otolith growth rate and embryonic growth with regard to temperature and suggested that no direct relationship existed between the two, but rather that the rate of a metabolic process governs the rate of otolith formation.

Arctic char (*Salvelinus alpinus*) is of high commercial and recreational importance, constituting a major element of the fish fauna of many lakes in northern Sweden. The Arctic char exists in a variety of morphological forms that have been of scientific interest for many years. Recent taxonomic characterization of the fish has lead to the suggestion of three distinct species based on gene frequencies and morphological and ecological characteristics (Nyman et al. 1981).

The fish material in our investigation were collected from "type-populations," N, F, and S-types, selected to represent each species (L. Nyman, pers. comm.). The possibility of back-calculating early life growth characteristics from otolith microstructures would be of great importance in understanding the ecology of the Arctic char complex. From the existing literature, we realized the need to investigate the conditions for back-calculation.

The present study was designed to examine the simultaneous influence of temperature and fish growth rate on otolith growth rate. Since temperature acclimation may influence the scope of growth (Elliott 1982), we designed the experiments to take into account the effects of earlier temperature regimes.

## Materials and Methods

### Experimental Design

Experiments on Arctic char fry were conducted from June through October 1984 in two stages. First, three different char types at two temperatures were followed in a long-term experiment (103 d) in six setups. Second, after 90 d of the ongoing first experiment, char fry were selected from each setup to be studied more individually under controlled laboratory conditions at six different temperatures while fed maximum food rations. These experiments ranged from 8 to 17 d.

### First Experiment

The first growth experiment at temperatures of 8 ± 1°C and 13 ± 2°C (± SD) was conducted at the Fishery Research Station at Älvkarleby from 13 June to 24 September 1984 to study long-term temperature acclimation on otolith growth. Char alevins hatched during April 1984 of the N, F, and S types were transported from the Fishery Research Station at Kälarne. Two groups of each type were selected consisting of about 150 individuals each. The fish were put into six 120-L troughs with a constant water flow (5–8 $L \cdot min^{-1}$). Light conditions were diffuse daylight and artificial light from 0700 to 1600. Fish were fed char pellets (Skretslet A/S No. 1), dispensed by automatic feeders at 20-min intervals from 0700 to 1600. The char were fed ad libitum. Measurements of weight and length were made initially and after 34, 65, 90, and 103 d. At the same time, a number of specimens (30, 30, 18, 32, and 62, respectively) were sacrificed for further analysis of otoliths.

### Second Experiment

The second growth experiment at six temperatures (8.0, 10.1, 12.4, 13.9, 15.9, and 18.8°C) was conducted at the Institute of Limnology, Uppsala University, during two periods, the first from 14 September to 1 October 1984 and the second from 2 to 10 October 1984. Generally, temperature variations ranged within ±0.2°, but on several occasions, short (less than 12 h) fluctuations of ±1° were recorded. During a single fault in the thermoregulatory system of the 8.0°C group, the temperature gradually fluctuated to a maximum of 12°C during a 30-h period. Fish taken from the first growth experiment were classified into size-class "small" or "large" dependent on whether these had grown at 8°C (small) or 13°C (large). The fish were marked individually using epidermal injections of alciane blue to make coded spot sequences. Seven groups of nine individuals (three of each char type) were put into separate 60-L aquaria. The fish were fed live *Chaoborus* larvae ad libitum (25% of fish wet $weight \cdot d^{-1}$). Added amount of food was noted and dead *Chaoborus* larvae were removed. Water for the aquaria was brought from Lake Björklinge-Långsjön, a calcareous clearwater lake in central Sweden. Water was continuously pumped through carbon filters (4 $L \cdot min^{-1}$) and aerated. Water was changed once during each experimental period. Light conditions were 8 h dark : 16 h light. For two groups of fish, 10.1°C "small" and "large," the experimental period was extended for another 8 d (2–10 October) combined with an increase in temperature, respectively, to 12.4 and 13.9°C. At the end of the experiment, all fish were sacrificed for analysis of otoliths.

### Otolith Preparation

Sagittae were removed from frozen individuals, rinsed in water, air-dried, and mounted in thermoplastic resin. The surface of the otoliths was ground and polished in aluminoxide paste to enhance visibility and to achieve maximal contrast and high transparency in the rostral structures.

The position of the otolith was adjusted so that the focal plane included the rostral and antirostral peaks as well as the periferal points of the transverse axis. The right sagitta was prepared for analysis by grinding and polishing both surfaces in the sagittal plane until a 20- to 30-μm-thick otolith shaving was produced. Often the otolith had to be flipped over several times before the preparation was sufficiently transparent for microscopic analysis.

In accordance with the nomenclature of Pannella (1980), all measurements were carried out in the sagittal plane, with each reading being defined by its angle (degrees) from the nucleus–rostrum axis and by its radius (micrometres) according to Fig. 1.

### Measurements of Otolith Increase

Measurements were conducted along different radii in the sagitta, the center of a radius being defined as the anterior primordium of the largest nucelus (Fig. 1).

Checkmarks in the appearance of dark bands are formed in salmonids by stress (e.g. confined space and transportation) or by temperature changes of several degrees during more than 2 h (H. Mosegaard, unpubl. data). Checkmarks were identified

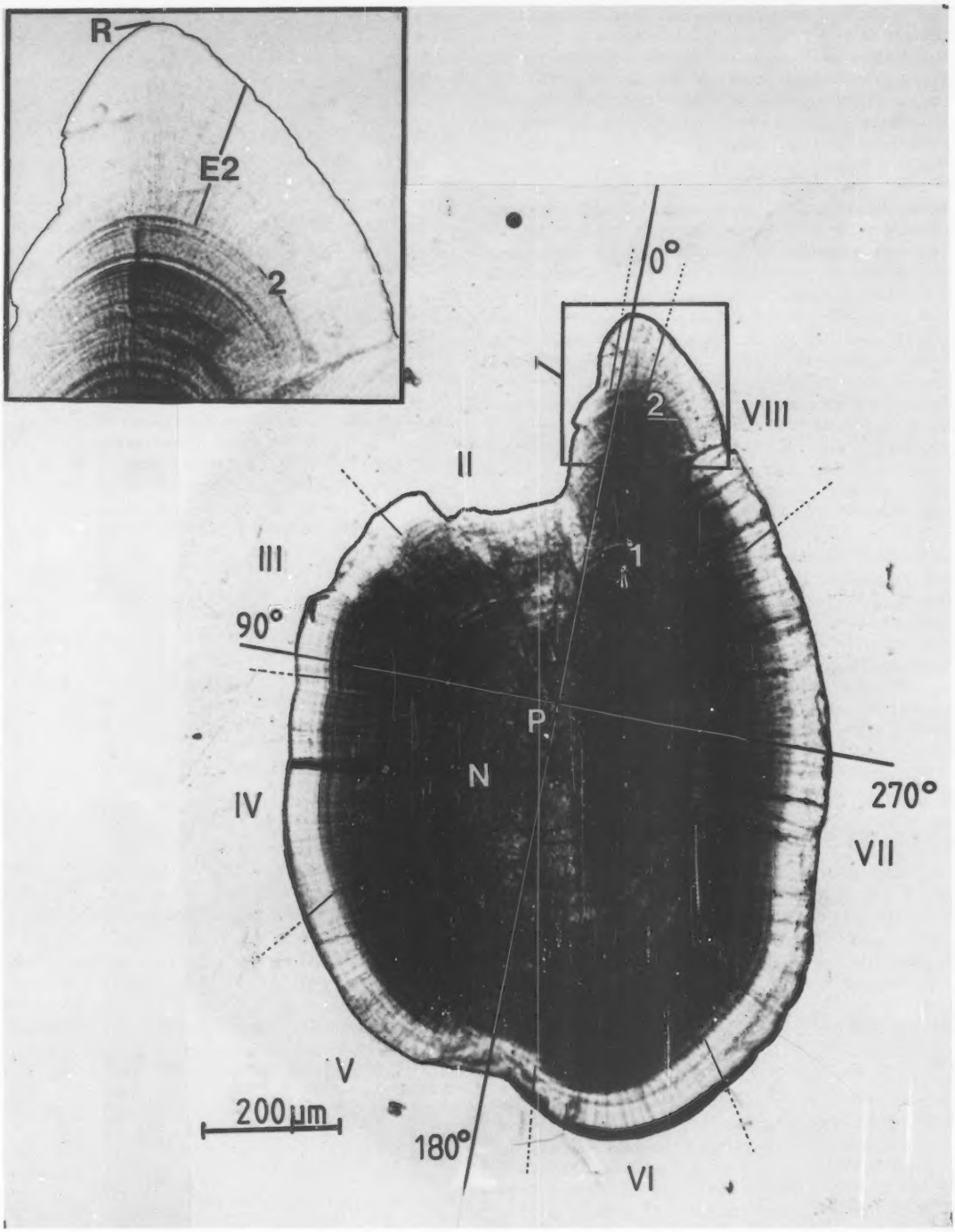


FIG. 1. Arctic char sagitta (ground and polished). Orientation of measurements along different radii is related to a system of polar coordinates. Readings are grouped into eight angular intervals (roman numerals). P, anterior primordium; N, nucleus; 1 and 2, checkmarks formed respectively at the start of the first and second experiments; R, rostral peak; E2, growth zone formed during the second experiment. Size-class "small," 18.8°C.

along each radius as originating from different treatments during the life of the fish. The width of the growth zone formed during an experiment was measured as the difference between the radius from the center to the checkmark of experiment conclusion (or otolith periphery) and the radius ending at the checkmark formed at the beginning of each experiment. The average daily radial growth rate, $\Delta R/\Delta t$, at a given angle is defined as the width of the experimental growth zone divided by the time elapsed (days). The relative radial growth rate, $\Delta R/\Delta t/R$, then, is the $\Delta R/\Delta t$ divided by the radius at the beginning of the experiment.

The width of the growth zone was measured within each of the eight angle intervals whenever the preparation of the otolith gave sufficiently clearly defined checks and an undisturbed peripheral outline.

Measurements of otolith structures were performed on ground and polished preparations using a compound light microscope with an optical tracing tube (Wild Herrbrugg Instruments) allowing a simultaneous view of the microscopic field and a digitizing tablet. When analysing structures, their location in relation to the otolith center and the first axis in the sagitta could be automatically recorded by manually placing the hair-cross cursor of the digitizing pad directly over the structure image and pressing the button.

A first processing of the data was conducted in an 8-bit microcomputer (Luxor-ABC800), where scaling and transformation into polar coordinates was executed. Two to four readings were conducted on each structure within each angular interval (Fig. 1).

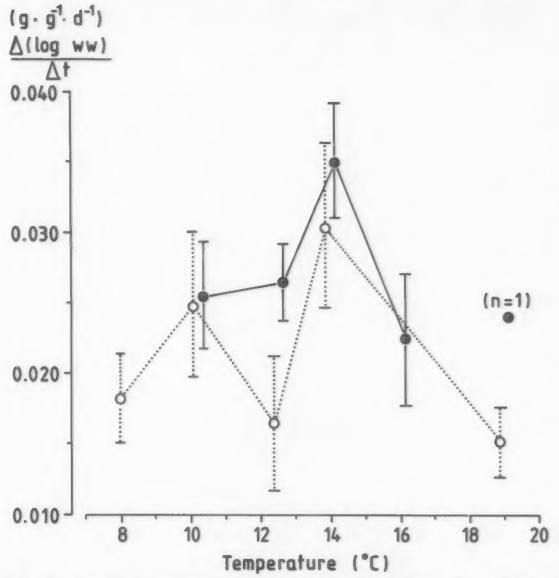


FIG. 2. Specific somatic growth rate, $\Delta(\log WW)/\Delta t \pm 2$ SE, of Arctic char fry with different growth histories at six temperatures calculated from differences between finish and start of log-transformed wet weights (g) divided by the length of the experimental period (d). ○, size-class "small" (103 d at 8°C); ●, size-class "large" (103 d at 13°C).

## Weight Increase of Otoliths

A possible importance in measuring the otolith growth rate in terms of weight increase was realized after finding differences in width of the radial growth zone between the two size-classes. There is implicitly a problem involved in estimating a weight increase of an individual otolith, due to the fact that otolith weighing is dependent on the sacrifice of the fish. Otolith weights may, however, be estimated from readings of otolith length at prior times in the individual's life using a regression analysis of otolith weight versus otolith length, and also including time and population characteristics.

However, the determined individual otolith weights cannot be used in the calculation of the weight at a prior time without the assumption that the weight of each individual otolith is related to its length by the regression equation and an individual factor determined by the final length and weight. Since this assumption cannot be checked by any length and weight measurements of otoliths, the weight increase should be considered a complex transformation of the increase in length but nevertheless, as we shall argue, a biologically reasonable one.

Since all otoliths from the second experiment were ground and polished before the importance of the weight increase was realized, a regression of otolith weights on otolith lengths, time, and size-class had to be based on char from the first experiment.

From the last three sampling dates in the first experiment, a total of 108 char fry otoliths were measured and weighed and a relationship between otolith weight (OW) and otolith length (OL), time (days), and size-class could be established

$$(1)\quad OW = e^{-14.6}\cdot OL^{1.87}\cdot e^{b\cdot time\cdot size\text{-}class} \qquad (R^2 = 0.961,\ CV = 5.8\%,\ n = 108)$$

where $b = 0.00302$ for size class "small" (long-term 8°C) and $b = 0.00358$ for size-class "large" (long-term 13°C). Since the otoliths probably grow in a nonisometrical way, depending on otolith size and age, this complex model including both size-class and time was chosen to describe the weight–length relationship for otoliths during the short-term experiments. However, the effect of size-class and time on otolith growth rate estimates was marginal.

The 95% confidence limits of individual weight estimates are slightly asymmetrical, due to regression analysis on log–log-transformed data. The upper limit range was found to be 14.5–15.5%, and the lower limit range was 12.5–13.5%.

Daily weight increase was then calculated from the difference between estimated otolith weight at the start and estimated weight at the end of the experiments based on measurements of the total length of otoliths at the start and end of the experiments. The estimates of otolith weights at the conclusion of the second experiment were calculated with some extrapolation of time (8 d for seven fish groups and 16 d for two groups), since the first experiment did not fully overlap with the second experiment.

In 11 cases where the posterior checkmark was obscured due to prolonged grinding, the width of this zone was estimated from a linear relationship between $\Delta R/\Delta t$ at 0° and $\Delta R/\Delta t$ at 180°:

$$\Delta R(180)/\Delta t = 0.21 + 0.69\cdot\Delta R(0)/\Delta t\ (R^2 = 0.86,\ p < 0.0001,\ CV = 10.9\%).$$

## Statistical Analyses

The data were statistically analysed using the SAS statistical programme package (SAS Institute Inc. 1982) on an IBM mainframe at Uppsala Data Central (UDAC). The otolith growth rate

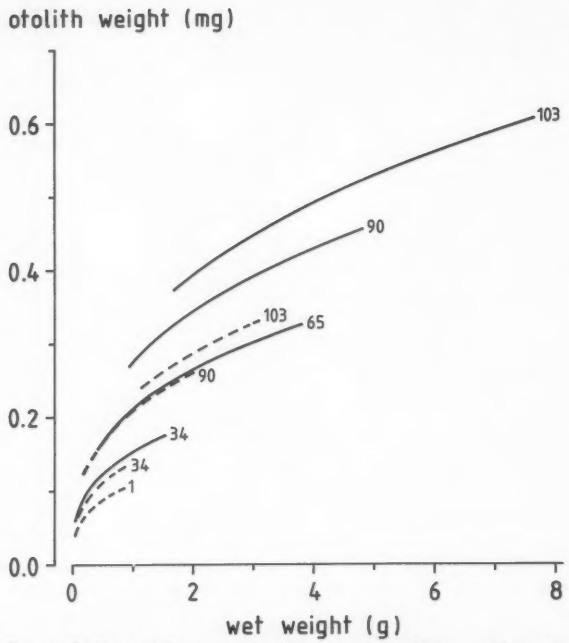


FIG. 3. Otolith weight vs. Arctic char wet weight of two experimental groups at five sampling dates (1, 34, 65, 90, and 103 d after start). Fitted regression lines are solutions to Equation 1: log OW = $-2.22$ + 0.326·log WW + time·$b_2$ (size-class) where $b_2$ = 0.0073 for fry reared at 8°C = size-class "small" (broken curves) and $b_2$ = 0.0103 for fry reared at 13°C = size-class "large" (solid curves).

as radial growth or estimated weight increase was treated as the dependent variable in a series of analyses using the generalized linear models (GLM) approach with various independent variables. Independent variables were primarily chosen to reflect important factors with a general impact on fish metabolism; however, factors arising from the experimental design were also investigated to determine how they possibly influence variation in otolith growth rates. Temperature ($T$) was tested as well as the transformation to natural logarithms, log $T$. Two parameters related to the individual fish were chosen: mean wet weight (determined as the mean of the initial and final weights for each experimental period) and mean growth rate, either as $\Delta WW/\Delta t$ or $\Delta(\log WW)/\Delta t$. Furthermore, the char type (N, F, or S) was considered as a covariate in a series of complex models to discover any differences in otolith growth rate implying a genetically founded effect. Finally, size-class was used as a covariate primarily to reflect a factor related to the experimental design.

## Results

### Somatic Growth Rate

In the first (long-term) experiment, growth in weight was exponential, with a mean specific daily rate of 0.022 at 8°C and 0.031 at 13°C. In the second experiment, increase in wet weight, $\Delta WW$, was significantly correlated with fish wet weight ($r$ = 0.87). We thus chose to use an exponential model to describe individual specific growth rate on a daily basis, $\Delta(\log WW)/\Delta t$. Mean values obtained for each temperature and size-class are shown in Fig. 2. We found that the response of specific growth rate to temperature was that of an optimum curve often found in fishes, although with a peculiar local minimum at 12.4°C in size-class "small" and no significant increase between 10.1 and 12.4°C in size-class "large."

### Otolith Size – Fish Size Relationships during the First Experiment

Analysis of otolith dry weight (OW) in relation to fish wet weight (WW) on five different sampling occasions showed that a marked contrast between the two groups evolved during the growing season. Assuming a linear relationship between log OW and log WW for each size-class at each sampling date, an ANCOVA gave the following equation:

$$\log OW = b_0 + b_1 \cdot \log WW + b_2 \cdot \text{time} \cdot \text{size-class} \qquad (R^2 = 0.96,\ CV = 8\%)$$

where $b_0 = -2.22$, $b_1 = 0.326$ ($p < 0.0001$), $b_2 = 0.0073$ (small), and $b_2 = 0.0103$ (large) ($p < 0.0001$)), the coefficient corresponding to size-class "large" being significantly larger than size-class "small." The groups at 13°C "large" developed larger otoliths than the groups at 8°C "small" relative to the same fish wet weight (Fig. 3). Ninety-five percent confidence limits of the predicted regression lines ranged from 2 to 6% of the predicted mean values.

### Microstructure Appearance

During the long-term experiments, otoliths from both 8 and 13°C groups showed clearly visible microstructures that could be detected from a checkmark formed at the date of transfer to the experimental troughs (1 in Fig. 1). Also, a very distinct checkmark was formed when the fish were transported to laboratory facilities at the limnological institute and again at transfer to individual temperature experiments (2 in Fig. 1).

Microstructures formed during the second experiment were rather diffuse compared with diurnal zones from the first experiment, with the exception of checkmarks formed on occasions when the fish were handled in the course of weighing and measuring them. Further, the transparency at the two highest temperatures was greater than at other temperatures in both short- and long-term experiments (E2 in Fig. 1).

### Growth Zone in Short-Term Temperature Experiments

Generally the zone was found to be formed all around the edge of the sagitta at the longitudinal-dorso-ventral plane, except at the groove in the anterior direction where the sulcus acusticus meets the edge. High correlations of mean daily radial growth rates were found between all eight angle intervals except area II; excluding II, the range of correlation coefficients was 0.46–0.93.

Analyses of covariance were performed on radial growth rate, $\Delta R/\Delta t$, as a function of temperature ($T$), class of radius ($A$) (grouped by intervals of angles see Fig. 1), and size-class ($S$) plus possible terms of interaction between these variables. From the results of the analysis of the equation

$$\Delta R/\Delta t = a_0 + a_1 \cdot T + a_2 \cdot A + a_3 \cdot S + a_4 \cdot T \cdot A + a_5 \cdot T \cdot S + a_6 \cdot A \cdot S + a_7 \cdot T \cdot A \cdot S,$$

variables contributing significantly to explanation of total variability were put together and reanalysed in the following equation:

$$\Delta R/\Delta t = a_0 + a_1 \cdot T + a_2 \cdot A + a_3 \cdot S + a_4 \cdot T \cdot A + a_6 \cdot A \cdot S.$$

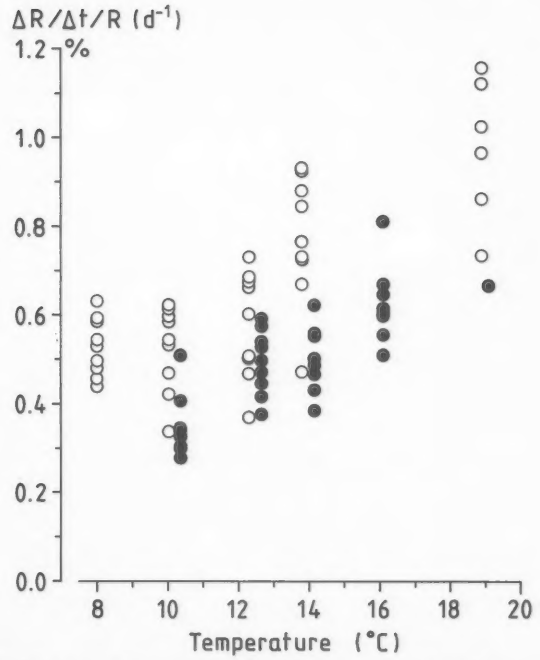


FIG. 4. Linear daily percentage growth along the rostral radius versus temperature of Arctic char fry with different growth histories. ○, size-class "small" (103 d at 8°C); ●, size-class "large" (103 d at 13°C).

Four variables, $T$, $T{\cdot}A$, $S$, and $A{\cdot}S$, were shown to be highly significant ($p < 0.001$) whereas radial group was significant ($p = 0.017$) and the intercept term $a_0$ ($p = 0.054$). These results indicate an interaction between the radial group and a linear form of the temperature effect, as well as between radial group and size-class. The coefficients corresponding to the different radii indicated a proportionally higher growth rate in the longitudinal direction at higher temperatures than in the tranverse direction with the same rise in temperatures.

The partial sum of squares for the interaction term $a_4{\cdot}T{\cdot}A$, however, was only significant when the interaction term between size-class and radial group was included as well as size-class ($a_3{\cdot}S + a_6{\cdot}A{\cdot}S$), a most peculiar result which may indicate several disturbing influences of intercorrelation between independent variables and a possible nonlinearity of temperature influence. Because of this finding, temperature was treated as a class variable in an ANCOVA, following the above procedure to select significant effects. Analysis of the form

$$\Delta R/\Delta t = a_0 + a_1{\cdot}T + a_2{\cdot}A + a_3{\cdot}S + a_4{\cdot}T{\cdot}A + a_6{\cdot}A{\cdot}S$$

showed no significant contribution from the interaction between radial group and temperature ($T$ as class variable) ($p = 0.32$). All other effects were highly significant ($p < 0.01$).

Therefore, we conclude that no systematic temperature-dependent differences in sagitta shape occur along with the growth in the studied fish sizes. The coefficients for individual groupings show, however, that the highest relative growth rates occur in the longitudinal direction and that the greatest change occurs in size-class "small."

We suggest that any radius will be representative when studying temperature effects on sagitta growth rate in juvenile char, although other factors, such as a low coefficient of variation in a particular area (radial group), would lead the investigator to choose this area for increment readings.

## Daily Growth Rate along the Anterior Radius

The relationship between mean daily growth along the rostral radius, $\Delta RO/\Delta t$, and the mean daily growth rate of the individual fish, $\Delta WW/\Delta t$, was analysed. Correlations with somatic and specific somatic growth rates were not significant, with $r$ values of $-0.04$ and $-0.06$, respectively.

When a correlation analysis of $\Delta R/\Delta t$ with temperature is conducted, a high $r$ value of 0.76 is found. Also the relative growth rate $\Delta R/\Delta t/R$ has a strong correlation with temperature ($r = 0.64$). When relative otolith growth rate $\Delta R/\Delta t/R$ is plotted against temperature, the data cluster into two groups which are strongly dependent on their respective size-class (Fig. 4).

Adding wet weight or somatic growth rate functions to models of $\Delta R/\Delta t$ dependence on temperature gave no significant contributions (measured as partial sums of squares) whereas these additions in models of specific radial growth rate were significant (Table 1). This result indicated that radial growth rate was to some extent dependent on the initial length of the radius. An ANCOVA showed that size-class affiliation added highly significant contributions to models describing the relationship between $\Delta R/\Delta t$ or $\Delta R/\Delta t/R$ and temperature (Table 1). Analysis of the residuals of the model, $\Delta R/\Delta t/R = i + a{\cdot}\text{temp} + b{\cdot}WW$, showed that the residuals from size-class "large" generally had lower values than size-class "small." This result indicates a lower growth rate along radii for size-class "large" compared with size-class "small" in relation to temperature and wet weight.

TABLE 1. Regression analyses of radial growth rate. $\Delta R/\Delta t$, and this measure in relation to radius at the start of the experiments, $\Delta R/\Delta t/R$. Significance of the partial regression coefficients: NS = not significant, $i$ = i intercept, $T$ = temperature, WW = fish wet weight, size-cl = two different size-classes from the first experimental period, reared at 8 and 13°C, respectively. Dependent variables on left-hand side; independent variables on right.

| Model | $i$ | $a$ | $b$ | $c$ | $R^2$ |
|---|---|---|---|---|---|
| $\Delta R/\Delta t = i + a{\cdot}T + b{\cdot}WW$ | NS | **** | NS | — | 0.58 |
| $\Delta R/\Delta t = i + a{\cdot}T + b{\cdot}WW + c{\cdot}$size-cl | ** | **** | ** | **** | 0.66 |
| $\Delta R/\Delta t = i + a{\cdot}T + b{\cdot}(\Delta(\log WW)/\Delta t)$ | NS | **** | NS | — | 0.58 |
| $DR/\Delta t = i + a{\cdot}T + b{\cdot}(\Delta(\log WW)/\Delta t) + c{\cdot}$size-cl | NS | **** | NS | ** | 0.63 |
| $\Delta R/\Delta t/R = i + a{\cdot}T + b{\cdot}WW$ | ** | **** | **** | — | 0.55 |
| $\Delta R/\Delta t/R = i + a{\cdot}T + b{\cdot}WW + c{\cdot}$size-cl | **** | **** | NS | **** | 0.69 |
| $\Delta R/\Delta t/R = i + a{\cdot}T + b{\cdot}(\Delta(\log WW)/\Delta t)$ | NS | **** | NS | — | 0.41 |
| $\Delta R/\Delta t/R = i + a{\cdot}T + b{\cdot}(\Delta(\log WW)/\Delta t) + c{\cdot}$size-cl | *** | **** | NS | **** | 0.70 |

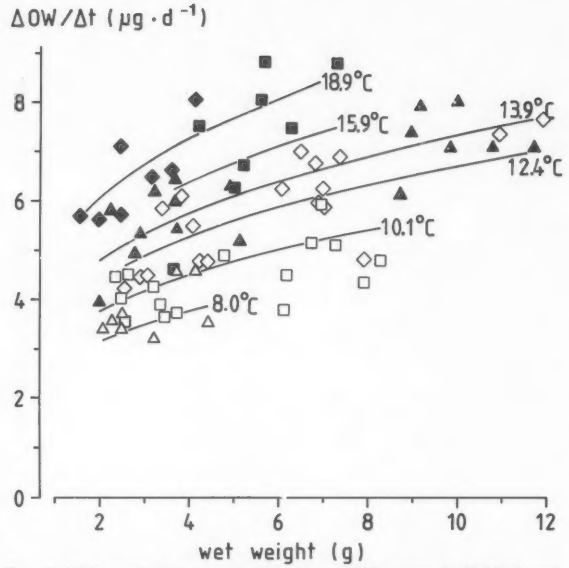


FIG. 5. Daily weight increase of sagitta otolith versus individual wet weight of Arctic char fry at each experimental temperature. Curves are drawn according to the solutions of the linear regression model $\log(\Delta OW/\Delta t) = -7.54 + 0.26 \cdot \log WW + 0.77 \cdot \log T$. $\Delta OW/\Delta t$ is estimated daily increase in otolith weight, WW is mean wet weight of the fry during the experiment, and $T$ is the mean of each experimental temperature. Lower and upper 95% confidence limits of individual predicted values are approximately −22 and +28%. Δ, 8.0°C; □, 10.1°C; ◇, 12.4°C; ▲, 13.9°C; ■, 15.9°C; ◆, 18.9°C.

Since otolith size, as length, rostral radius, and weight, was found to be greater in relation to fish size in size-class "large," we suggest that the generally lower growth rate of the otolith radius in fish from this size-class might be due to the relatively larger otolith size at the start of the second experiment and that the apparently different growth rates in the (one-dimensional) radius measure might correspond to equal growth rates, when calculated on a (three-dimensional) volume or a weight basis.

### Models of Daily Otolith Weight Increase

The mean daily rate of increase in sagittal weight, $\Delta OW/\Delta t$, was used as the dependent variable in a GLM approach with a number of independent variables.

As in analysis of otolith growth rate, the independent variables temperature, fish wet weight, and somatic growth rate, as well as the covariates char type and size-class affiliation, were considered. Individually, temperature was the most important factor ($R^2 = 0.49$–$0.51$, $p < 0.0001$) but wet-weight also explained a large part of the variation in otolith weight increase ($R^2 = 0.31$–$0.32$, $p < 0.0001$). On the other hand, fish growth rate explained little and specific somatic growth rate almost no variation in otolith weight increase ($R^2 = 0.15$–$0.19$ and $0.01$, respectively).

An ANOVA showed significance in explaining otolith growth rate by the division of the material according to size-class affiliation ($R^2 = 0.35$–$0.38$, $p < 0.0001$). Finally we found that char type and factors primarily connected with the experimental design were not significant in explaining variation in rate of otolith weight increase.

We decided to model otolith growth rate expressed as a daily increase in weight as a function of temperature and fish wet-weight. ANCOVA of linear or log-transformed values of the dependent and independent variables $\Delta OW/\Delta t$, $T$, and WW gave highly significant partial sums of squares ($p < 0.0001$) whereas adding size-class to the models resulted in no significant contributions in any of the cases, either as additive or interaction terms (probabilities ranging from 0.07 to 0.98 that size-class effects were random).

Due to the slight dependence of the residual variance on both temperature and weight in the linear form, we chose log transformation of all variables in the expression of otolith weight increase in relation to temperature and fish wet weight, with the solution of the parameters resulting in the following model:

$$(2)\quad \Delta OW/\Delta t = 5.33 \times 10^{-4} \cdot T^{0.7654} \cdot WW^{0.2621} \qquad (R^2 = 0.80,\ CV = 2.4\%).$$

The observed and predicted values of otolith growth rate of this model are shown in Fig. 5. Ninety-five percent confidence limits of individual predicted values were slightly asymmetrical due to the log–log transformations. Expressed as a percentage, limits were respectively 22 below and 28% above predicted values.

## Discussion

### General Concepts of Otolith Formation

Back-calculation of fish size at different ages from dated structures in hard parts of fishes is a highly effective instrument in fisheries biology. It does, however, presuppose two important conditions. The first is that the structures in question can be age determined with adequate precision; the second is that a unique correspondence exists between the size of the hard part and fish size at a given age and, further, that an unbiased estimate from this correspondence can be obtained.

It is captivating to think of otolith microstructures as a day-to-day record of individual fish growth rates. Although pertinent literature presents several examples of an uncoupling between otolith and fish growth rates (Brothers 1981; Marshall and Parker 1982; Wilson and Larkin 1982; Campana 1983; Rice et al. 1985). The general concept is that such a relationship exists (Ralston 1976; Marshall and Parker 1982; Neilson and Geen 1982, 1985; Miller and Storch 1984; Volk et al. 1984; Campana and Neilson 1985). Consequently, a number of authors have used otolith microstructures to back-calculate either fish size or estimated relative growth rates on a daily basis (Ralston 1976; Struhsaker and Uchiyama 1976; Methot and Kramer 1979; Worthmann 1980; Rosenberg and Haugen 1982; Wilson and Larkin 1982; Victor 1982; Ralston and Miyamoto 1983; Penney and Evans 1985).

### Deviations from the General Concepts of Otolith Formation

The present study exhibits several departures from the generally accepted view that otolith incremental widths reflect a running average of fish growth rates (Campana and Neilson 1985). The most essential points are as follows: (1) the response of otolith growth rate to increasing temperature is totally different from the optimum curve of somatic growth rate, such that otolith growth rate continues to increase above temperatures of maximum somatic growth rate; (2) the variation in otolith growth rate, compensated for by variation in temperature, is better explained by fish size than by fish growth rate; and (3) the explanation of the variation of otolith growth rate being higher when the growth rate is expressed as weight increase per

day than when being expressed as increase in radius per day is due to the fact that the latter measure is influenced by earlier otolith growth.

The point that otolith growth rate departs from fish growth rate by steadily increasing with increasing temperatures indicates that otolith growth may be related to a metabolic expression of the fish.

Although circadian changes in metabolic activity have been considered responsible for variation in the otolith calcium deposition rate (Campana and Neilson 1985), the considerations have not been extended to the overall control of the otolith formation rate. Temperature is known to strongly influence various parts of the metabolism in fish and thereby indirectly somatic growth rate to a very high degree (Elliott 1982). Nevertheless, reports of systematic investigations on the effect of temperature are not found in the relevant literature (Mosegaard and Titus 1987).

The results presented are based on analyses of various models of the otolith growth rates as functions of fish parameters and environmental factors. In the search for an assessable model of otolith growth rate, importance was put upon finding a mathematically simplistic expression with maximum generality and biological substance. Therefore the elimination of the size-class effect from the models by expressing otolith growth rate as daily weight increase instead of radial increment per day was considered important. It could be argued that the effect of size-class on otolith radial growth rate is a long-term temperature acclimation effect, but since this effect did not turn out to be significant when otolith growth rate was calculated on a weight basis, we would prefer to regard the differences in radial growth rates between size-classes as the differential effect of a weight increase on otoliths of the two different size categories. Thus, the same increase in weight would produce a larger incremental width along a radius on a small otolith than on a large otolith. In the following discussion, only otolith growth rate as the weight increase expression will therefore be used when not otherwise expressly stated.

## Different Otolith and Somatic Growth Rate Responses to Temperature

Temperature is documented to have a strong influence on incremental width in otoliths (Brothers 1981; Marshall and Parker 1982; Neilson and Geen 1982; Campana 1984). The importance of temperature as a governing factor for otolith growth rate was also shown in this study. An increasing otolith growth rate was found for all temperatures from 8°C to well above the 14°C maximum for somatic growth rate. This discrepancy between otolith and somatic growth over a broad temperature range is in agreement with a study on yolk-sac fry of Arctic char and brown trout (*Salmo trutta*) (Mosegaard and Titus 1987). Besides this study, we are not aware of any other reports on the effects of hyperoptimal temperatures on otolith growth.

Other writers, however, have observed uncoupling between otolith growth and fish growth under conditions of starvation (Brothers 1981; Marshall and Parker 1982; Campana 1983).

Somatic growth rate is often found to have a maximum response at some intermediate temperature within the range of tolerance (Webb 1978). This is also the case with Arctic char (Swift 1964; Jobling 1983). The temperature response of otolith growth, however, is not easily explained by any known growth function. The decline in somatic growth rate at high temperatures has been explained as the respiration rate being progressively higher than anabolic processes with rising temperatures (Webb 1978). Being a structurally extracellular process, otolith formation may be expected to behave in a fashion that is different from that of somatic cellular growth.

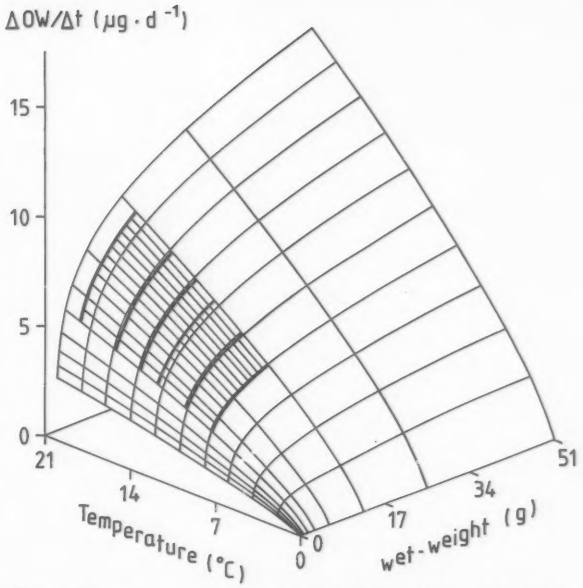


FIG. 6. Three-dimensional representation of the sagitta growth rate in Arctic char as a function of temperature and fish wet weight predicted by the model $\Delta OW/\Delta t = 5.33 \times 10^{-4} \cdot T^{0.7645} \cdot WW^{0.2621}$. The range in variation of experimental conditions is marked by heavy lines.

If $Q_{10}$ values of otolith growth rate are calculated in a number of models over the interval of 5–15°C, values in the range of 1.7–2.0 are found. Compared with the corresponding value of standard metabolism of 2.3 (Brett and Groves 1979), otolith growth rate is considerably lower. Residual analysis, however, reveals that a possible leveling out of otolith growth rate at the highest temperature might occur, a result also found by Mosegaard and Titus (1987). This result in combination with the transition to a more transparent otolith formation suggests that the mechanism of otolith formation at the highest temperatures might be different from that at low and intermediate temperatures. Thus, if otolith growth rates at 18.8°C are excluded from the models, $Q_{10}$ values in range 2.0–2.3 are found, much more in line with the temperature response of standard metabolism.

## Influence of Fish Parameters on Otolith Growth Rate

Metabolic rates in relation to wet weight in salmonids can be described as a power law (allometric equation), with the exponent lying in the range of 0.7–0.85 for standard metabolism (Elliott 1982) and with even higher exponents for small fishes (1 for 0.85- to 4-g *Salmo gairdneri*; Smith et al. 1978). Routine metabolism at low temperatures (2 and 6°C) for Arctic char was shown to have weight exponents of about 1 (Holeton 1973). These exponents for energy requirement are considerably higher than the fish weight exponent of 0.26 for otolith growth rate. Since calculations show that otolith growth with increasing fish size is at least not underestimated, the weight exponent cannot well be much higher, and otolith growth rate might not be proportional to metabolic requirements of the entire fish, but rather

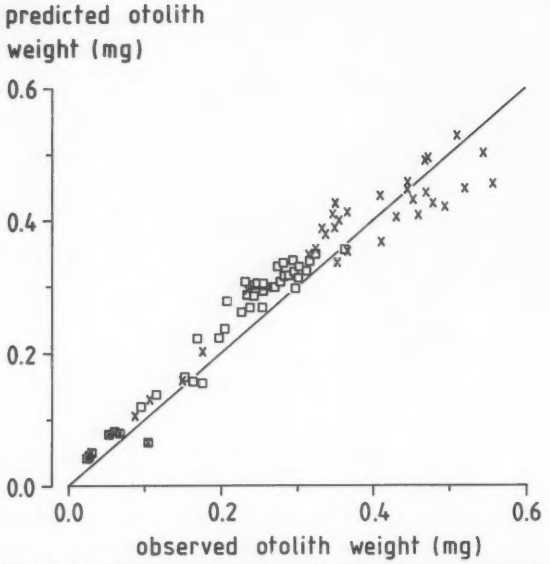


FIG. 7. Predicted versus measured otolith weights from Arctic char fry at two different temperatures. Predictions are made from Equation 3 on otoliths from the first experiment:

$$(3)\quad OW(t) = \frac{5.33 \times 10^{-4} \cdot T^{0.7654} \cdot WW(0)^{0.2621}}{0.2621 \cdot g} \times \cdot(e^{0.2621 \cdot g \cdot t} - 1) + OW(0).$$

□, size-class "small" (reared at 8°C); ×, size-class "large" (reared at 13°C). The line denotes the 1:1 expected relationship.

a function of some metabolic scope that has lower increase with body size than total metabolism.

The brain of the fish, being a static cell population, develops relatively slower than any other parts of the body (Weatherley 1972), so an adaptation of the inner ear to this pattern would be reasonable.

Although the weight exponent is not comparable with exponents from metabolic rates, the otolith growth rate "reacts" in other ways in a similar fashion. The otolith growth rate increases with higher temperatures and fish weight, the weight exponent decreases with decreasing nutritive level, but even at starvation, growth remains positive for a considerable time (Marshall and Parker 1982; Campana 1983; Rice et al. 1985; Mosegaard and Titus 1987). Compared with an exponent of standard metabolism of about 0.75, the exponent of the otolith growth rate is lower by a factor of 0.5. On the other hand, if the calcium–secreting tissue is related to the weight of the otolith as a surface layer relates to a solid body (with an exponent of 1.5), the exponent for this tissue will be 0.75, quite in accordance with the magnitudes of metabolic weight exponents. Thus, the overall metabolic rate could govern the excretion of the calcium complex, affecting the otolith growth rate with a low exponent in the wet weight part of the expression, but otherwise following the metabolic activity, influenced by temperature, feeding, and other activity cycles.

Although the temperatures were lower, Holeton (1973) did not consider the weight exponent of the routine metabolism to be temperature dependent in Arctic char. Generally the weight exponents for metabolic expressions in salmonids are not found to be temperature dependent (Elliott 1982). Consequently, without a more extensive investigation of both temperatures and fish sizes, the more complex models with temperature-dependent weight exponents of otolith growth rate are not considered.

## Validation of the Otolith Growth Rate Model

From the model of the char sagitta growth rate (milligrams per day) (Equation 2; Fig. 6), a calculation of the otolith weight – fish wet weight relationship was performed according to the observed growth rates of fry at 8 and 13°C in the first experiment. If the growth in wet weight is assumed to be exponential and the instantaneous otolith growth rate is approximated by the determined difference equation, then the otolith weight may be calculated by integrating following differential equation:

$$dOW/dt = \Delta OW/\Delta t = 5.33 \times 10^{-4} \cdot T^{0.7654} \cdot WW^{0.2621}$$

which will have the following solution for time $t$:

$$OW(t) = 5.33 \times 10^{-4} \cdot T^{0.7654} \cdot \int_0^t WW(0)^{0.2621} e^{0.2621 \cdot g \cdot x}\, dx + OW(0)$$

$$(3)\quad OW(t) = \frac{5.33 \times 10^{-4} \cdot T^{0.7654} \cdot WW(0)^{0.2621}}{0.2621 \cdot g} \times \cdot(e^{0.2621 \cdot g \cdot t} - 1) + OW(0)$$

where $g$ is the exponential growth rate of the fish, WW(O) is the wet weight of the fish at the start of the experiment ($t$ = 0), and OW(O) is the estimated weight of the otolith at the same time.

This model was used to predict the otolith size at different dates of sampling in the long-term experiments. The predicted versus the observed otolith weights are shown in Fig. 7. The model was found to predict otolith weight for each fish reasonably well. However, an overestimation was found in most cases, the effect being more pronounced in the 8°C groups. Considering the possibilities of errors affecting the result, such as differing growth rates within temperature groups and the effect of variation around the mean temperature, this rough estimation of otolith growth seems sufficiently satisfactory to verify the model describing otolith growth in juvenile char at high food rations as a power function of fish wet weight and temperature. No long-term experiments were conducted on temperatures above optimum for somatic growth, so validation of high-temperature, long-term effects could not be performed.

The model was also used to predict otolith size corresponding to age and fish weight in a population of rapidly growing large Arctic char from southern Sweden (Lake Unden). The population was assumed hypolimnic, with mean temperatures of 7°C during summer and 3°C during winter. Estimated otolith weights from mature individuals in the range of 6–12 yr and 0.7–4.2 kg wet weight were somewhat (10–30%) above the measured values (H. Mosegaard, unpubl. data). These applications show that extrapolation of the model to fish weights far beyond both ends of the range of experimental weights predicts otolith weight values in the right order of magnitude. Overestimation, however, is found in both applications and might indicate that otolith growth rates are somewhat reduced when fish growth rates are not at maximum.

## Applications of the Model to Specific Cases in the Literature

During the first (long-term) experiment, different exponents were found relating otolith weight to fish weight at the two temperatures. This result would be expected, considering the

exponential growth rates found in the experiment. The exponents ($g$) of 0.022 at 8°C and 0.0305 at 13°C, put into a model of the form of Equation 3, would result in this type of relationship.

Wilson and Larkin (1982) showed when back-calculating weights at a previous date a nonrandom departure from linearity from a general regression of log fish weight on log otolith radius, which they suggested could be explained by possible abrupt changes in otolith growth or body weight. In their study, temperature was not monitored, but information was given that temperature rose from 6 to 13°C during the experiment. Since two growth periods were considered in the same study, one would expect a higher mean temperature at the later period, possibly giving rise to the same type of deviation in the otolith growth – fish growth relationship as we found in our long-term study. Our study shows the importance of temperature in determining the otolith size – fish size relationship, and its effect should be considered in all events where back-calculation from otoliths is performed.

Volk et al. (1984) presented evidence in favor of a dependence of otolith zone width on growth rate of chum salmon (*Oncorhynchus keta*) at different prey rations. A regression coefficient of 1.05 was found, with mean increment width as a function of log percent growth rate. If the otolith growth rate (described by Equation 2) and the relationship between otolith length and otolith weight (described by Equation 1) from the long-term experiments on char fry are applied to calculate a mean daily incremental width for the chum salmon in Volk et al. (1984), then a regression equation of $Y = a + b \cdot \log(I\text{-width})$, with $b = 0.52$ and a correlation coefficient of $r = 0.97$, would be found, provided the fry are growing exponentially and the otoliths have the same growth rate dependence on temperature and wet weight as do char. By applying Equation 3 over short periods of time (less than 100 d), otolith growth will almost be linearily correlated with fish growth within a reasonable interval of exponential growth rates, from negative to 5% daily weight increase, at a constant temperature due to the dependence of otolith growth rate on fish size. The higher slope ($b$) found in the chum study (Volk et al. 1984), however, indicates that fish growth rate might influence otolith growth in some other way than is accomplished through the differential effect of the fish weight.

The idea that some metabolic activity, and thus an increasing function of fish size and temperature, is responsible for the otolith growth rate is supported by this finding, since metabolism is "regulated" by acclimation and might be reduced by low food conditions (Staples and Nomura 1976).

Preliminary results indicate that a general relationship between otolith growth rate and temperature and fish weight, illustrated by Equation 2, might hold within a range of food levels and during short periods of starvation as well (H. Mosegaard, unpubl. data).

While investigating haddock (*Melanogrammus aeglefinus*) populations, Templeman and Squires (1956) found higher otolith weights at a given fish size in slow-growing than in fast-growing populations. Given the same temperatures, Equation 2 would predict that both age and somatic growth rate would determine the otolith weight – fish weight relationship, thereby giving a plausible explanation of the findings in Templeman and Squires (1956). If, however, temperatures vary between populations, a more complex pattern will emerge, since the effect of an increase in temperature (below optimum), at high food levels, will increase both somatic and otolith growth rates. The otolith weight – fish weight relationship will thus depend on the actual temperature response of somatic growth rate at a particular food level.

Important restraints are put on back-calculations from daily otolith rings, provided the presented results in this study are general. The uncoupling between otolith growth rate and daily somatic growth rate may be disappointing; however, the determined relationship with temperature and fish size may partially compensate for this uncoupling, provided there is a knowledge of food accessibility. Further, if a functional correspondence to a basal metabolic rate can be established, a method could be developed to help estimate energy budgets for fish populations in nature.

## Acknowledgements

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# Physiological Disturbances in Fish Living in Coastal Water Polluted with Bleached Kraft Pulp Mill Effluents

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Andersson, T., L. Förlin, J. Härdig, and Å. Larsson. 1988. Physiological disturbances in fish living in coastal water polluted with bleached kraft pulp mill effluents. Can. J. Fish. Aquat. Sci. 45: 1525–1536.

An extensive trial to examine physiological and biochemical changes in perch (*Perca fluviatilis*) inhabiting coastal waters polluted by bleached kraft mill effluents (BKME) has been carried out. The investigations were performed at four different times of year. Fish from a reference site and from sampling sites 2, 4.5, 8, and 10 km from the discharge point were examined. The results of the investigation show profound effects of BKME on several fundamental biochemical and physiological functions. Typical symptoms in perch from the polluted areas were reduced gonad growth, enlarged liver, and very strong induction of certain cytochrome P-450-dependent enzyme activities in the liver. Elevated levels of ascorbic acid in liver tissue and abnormal carbohydrate metabolism reflect the effluent's ability to cause metabolic disorders. Marked effects on the white blood cell pattern indicate a suppressed immune defence. Alterations in the red blood cell status and in the ion balance suggest that the demand for oxygen by certain tissues was increased and that gill function was impaired, respectively. The toxic effects were most pronounced in fish living up to 4.5 km from the discharge point, but some disturbances (e.g. cytochrome P-450 induction, reduced gonad size, and hematological alterations) were observed in fish caught as far as 8–10 km from the kraft bleach plant.

Les auteurs ont réalisé une expérience détaillée afin d'étudier les modifications physiologiques et biochimiques présentées par des perches fluviatiles (*Perca fluviatilis*) dans des eaux côtières polluées par des effluents d'usine de production de pâte kraft blanchie. Les études ont été effectuées à quatre moments de l'année. Des poissons prélevés à un site témoin ainsi qu'à des sites situés à 2, 4, 5, 8 et 10 km du point de rejet ont été examinés. Les résultats montrent que les effluents ont eu des effets marqués sur plusieurs fonctions biochimiques et physiologiques fondamentales. Les symptômes types des perches des sites pollués comprenaient une croissance réduite des gonades, une hypertrophie du foie et une très forte induction de l'activité de certains enzymes du foie dépendants du cytochrome P-450. Les teneurs élevées d'acide ascorbique dans les tissus hépatiques et le métabolisme anormal des sucres reflètent la capacité des effluents à perturber le métabolisme. Des anomalies marquées des leucocytes indiquaient une perte de défense immunitaire. Des perturbations touchant les érythrocytes et l'équilibre ionique portent à croire, respectivement, à une augmentation de la demande d'oxygène de certains tissus et à une perturbation de la fonction branchiale. Les effets toxiques étaient plus prononcés chez les poissons se trouvant dans la zone des 4,5 premiers kilomètres du point de rejet, mais certaines anomalies (p. ex. : induction du cytochrome P-450, réduction de la taille des gonades et perturbations hématologiques) ont été observées chez des poissons capturés à 8 ou 10 km de l'usine.

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To satisfy our demand for clean, white paper, large amounts of chemicals are employed in industrial pulp bleaching processes. Effluents from such pulp mills drain into rivers, lakes, or coastal waters and are potentially harmful to aquatic life. It is therefore important that the impact of bleached kraft mill effluents (BKME) on the aquatic environment is evaluated. To do so, however, requires that sensitive and simple indicators are developed for assessing and predicting effects of BKME on animal populations.

BKME exposure disturbs several fundamental physiological and biochemical functions in fish (Hutchins 1979). Since changes in such functions precede the onset of more serious effects at the population and community levels, it has been proposed that biological monitoring programs incorporate physiological and biochemical indicators for routine prediction of toxic effects in the receiving body of water (Larsson et al. 1985; Förlin et al. 1986; Payne et al. 1987).

In a Swedish research project, Environment/Cellulose, an extensive trial is underway to examine the usefulness of biochemical and physiological parameters as indicators of health on fish exposed to BKME. In an introductory laboratory study, the long-term effects of BKME on the carbohydrate metabolism

and hepatic xenobiotic biotransformation enzyme in fourhorn sculpin (*Myoxocephalus quadricornis*) were investigated (Andersson et al. 1987). In the same laboratory study, the effects of BKME on the erythrocyte and leucocyte status, ion balance, and vertebral structure were investigated (Härdig et al. 1988). A third report from this experiment deals with the effects of BKME on the mechanical and biochemical characteristics of the vertebrae (Mayer et al. 1987).

The present report describes the first large-scale field investigation using biochemical and physiological response parameters to detect and diagnose disturbances in fish inhabiting waters receiving kraft pulp mill effluents. Perch (*Perca fluviatilis*) were sampled at various distances from the effluent outlet in an attempt to estimate the extent of the area surrounding the mill influenced by the BKME. Furthermore, the study was performed at four different times of the year to detect any seasonal variations in the toxic response. Parallel studies investigated fish species composition, fish growth, gonadal development, and the occurrence of external pathological alterations and of ectoparasites (Neuman and Karås 1988; Sandström et al. 1988; Lindesjöö and Thulin 1987).

## Materials and Methods

The study was conducted in the vicinity of a kraft pulp plant (Norrsundet Bruk AB) situated on the coast of the Gulf of Bothnia (Baltic Sea) north of Gävle (Fig. 1). Chlorine (85%) and chlorine dioxide (15%) are used as bleaching chemicals. The pulp plant uses pine and spruce wood and the bleaching process includes an introductory stage with oxygen bleaching and a subsequent bleaching in two parallel production lines (C85 + D15)(EO)D or (C85 + D15)(EO)DED, respectively. These bleaching sequences began during 1982–83. Regulations limit production to 240 000 tons of bleached kraft pulp per year. During 1984, the pulp mill discharged 51 tons of chemical oxygen demand (COD) per day into the receiving water. This emission rate decreased steadily to about 36 tons per day in 1985. Consumption of elementary chlorine was reduced by various internal measures from about 12 000 tons in 1983 to about 8700 tons in 1984 and 6800 tons in 1985. The effluent is diluted about 40 times with brackish water and pumped into the receiving water. The plume of the BKME describes a dilution gradient 10-km northeastward and then turns south into a gyre due to the southward main current. A more thorough description of the pulp plant, the hydrology and biology of the receiving water, and the chemical characterisation of the released BKME is given elsewhere (Södergren et al. 1988). No other industrial or municipal discharges are present in the investigated area.

Perch ranging in weight between 100 and 400 g were caught with gillnets at four different times (May 1984, August 1984, October 1984, and September 1985). In May, August, and October 1984, three sampling sites, 2, 4.5, and 8 km from the plant, were used (Fig. 1). At the three innermost sites (2, 4.5, and 8 km), dilution of the pulp mill effluent has previously been

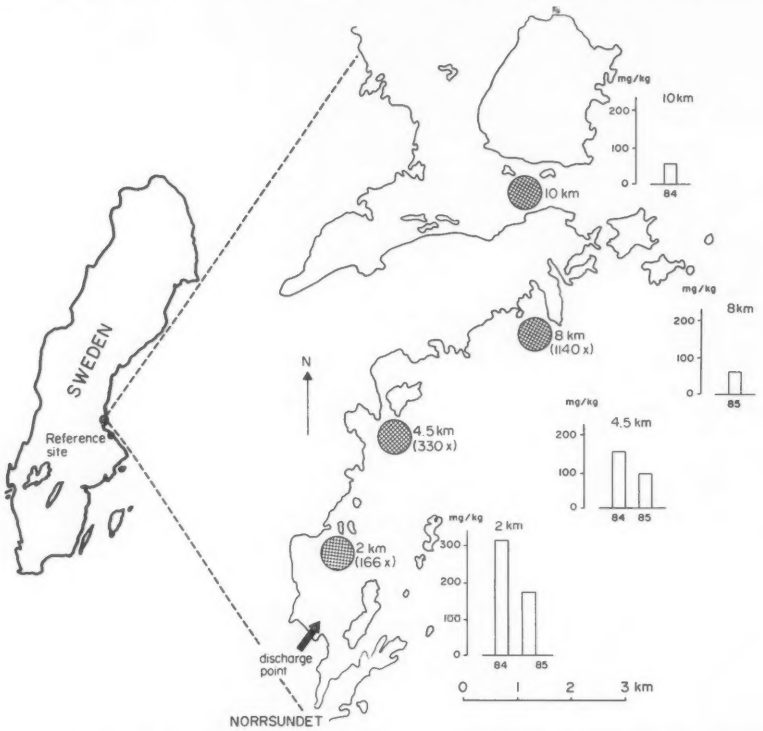


FIG. 1. Sampling stations for perch near a kraft pulp mill. Bars show the levels of EOCL in perch caught at the sampling stations polluted with BKME. EOCL analyses were performed on pooled muscle samples from 3–18 female perch ranging in length between 17 and 25 cm. Numbers in parentheses are the estimated dilution factors for the discharged waste water. The control site (Reference cite) was situated 120 km south of the kraft pulp mill.

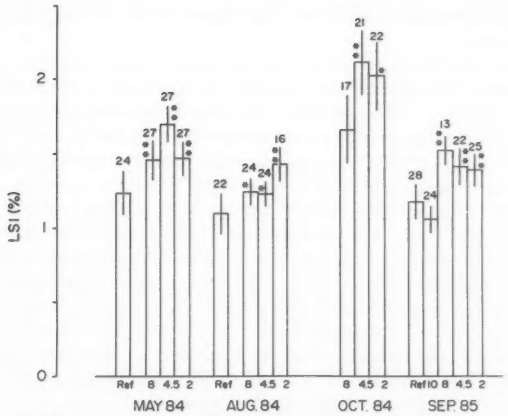


FIG. 2. Liver size, expressed as liver somatic index (LSI), in perch from the control site (Ref.) and from sites polluted with BKME. Polluted sites are situated 8, 4.5, and 2 km from the mill outlet. Bars give mean ± SD. Number of fish is indicated above the bars. Differences between fish caught at the control site and fish caught at the polluted sites were statistically significant at $*p < 0.05$ and $**p < 0.01$. In October 1984, when no control fish were obtained, levels of significance refer to differences between fish caught at the two innermost sampling sites (2 and 4.5 km from the outlet) and those caught 8 km from the outlet.

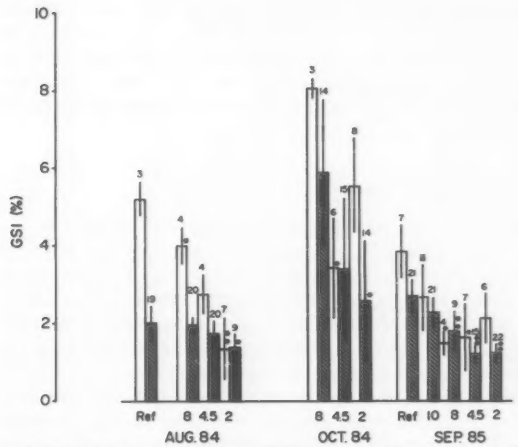


FIG. 3. Gonad size, expressed as gonadosomatic index (GSI), in male (open bars) and female perch (hatched bars) from the control site and from sites polluted with BKME. See legend to Fig. 2 for further explanations.

estimated to be about 166, 330, and 1140 times, respectively (Anonymous 1985) (Fig. 1). In September 1985, an additional site 10 km from the plant was sampled, which represented a less polluted area along the pollution gradient around the pulp mill. According to the fish population studies the perch is a stationary species and the effects seen should reflect the situation in that area (Neuman and Karås 1988). Control fish were caught at an unpolluted site, situated 120 km south of the kraft pulp mill, at each of the various sampling times except for October 1984 when no fish were obtained. The receiving water is shallow with a maximum depth at the sampling stations of 10 m. The receiving water has a steady salinity of 0.5%. The dissolved oxygen content in the water was measured at the sampling sites in August 1984 and was found to be close to saturation levels (9.4–10.1 mg/L). The water temperature at the different sites ranged between 8.0 and 11.5°C in May 1984, 16.5 and 17.5°C in August 1984, and 7.0 and 8.5°C in September 1985, and in October 1984 the temperature was 8.5°C at all sampling sites.

Before sampling, the fish were kept in wooden fish chests for 3 d. No mortalities were seen during holding. Haux et al. (1985) found that using a 3-d recovery period in fish chests, the physiological parameters affected by capture stress were stabilized and could be reliably assessed in perch.

The samplings of blood, liver, and muscle tissues were performed according to a standardized routine procedure previously described by Dave et al. (1975). Blood was taken from the caudal vessels by a heparinized syringe and needle. Hematocrit (Ht) was determined within 5 min after sampling. Blood for determining hemoglobin (Hb) content and red blood cell (RBC) counts was preserved on ice and analyzed within 24 h. Methemoglobin was determined according to Härdig and Höglund (1983) on RBC separated from the plasma. To determine the number of different types of white blood cells (WBC), blood smears were made on slides, air-dried, and cells counted after staining the smears by the Pappenheim method (Sandoz Ltd. 1973). δ-Aminolevulinic acid dehydratase (ALA-D) activity in the blood was measured according to the method of Johansson-Sjöbeck and Larsson (1978). Blood plasma levels of sodium, potassium, chloride, calcium, and magnesium ions were measured according to methods described by Haux and Larsson (1979). Blood glucose and lactate content and liver and muscle glycogen content were estimated as outlined by Dave et al. (1975). Ascorbic acid content in the liver was measured according to the method of Zannoni et al. (1974). Total bilirubin levels in blood plasma were measured by using Boehringer Mannheim test combination. Liver tissue used for assaying enzymes involved in xenobiotic metabolism was quickly frozen in liquid nitrogen according to Förlin and Andersson (1985). UDP glucuronosyltransferase activity towards *p*-nitrophenol and 7-ethoxyresorufin-*O*-deethylase activity was measured as described by Andersson et al. (1985). All enzyme activities were measured at 20°C. The liver and gonad somatic indices (LSI and GSI, respectively) were calculated according to the following formula: organ weight (grams) × 100/body weight (grams).

The data were analyzed by one-way analysis of variance followed by Duncan's new multiple-range test (Winer 1971) using the data program MULREG 800 on the ABC microcomputer system (Luxor, Sweden). Statistically significant differences between groups have been indicated at $P < 0.05$ and $P < 0.01$. Fish from sampling sites in the vicinity of the pulp mill were compared with reference fish from the same sampling period except for October 1984, when fish from the two innermost polluted sites (2 and 4.5 km) were compared with those caught 8 km from the pulp mill.

## Results and Discussion

The muscle levels of extractable organic chlorine (EOCL) (analyzed by the Center for Industrial Research, SI, Oslo, Norway) were measured in perch caught at 2, 4.5, 8, and 10 km from the mill in May 1984 and in September 1985. The results of these analyses clearly demonstrated that levels of EOCL in perch decreased as distance from the mill increased from 2 to 10 km (Fig. 1), confirming the existence of a well-defined pol-

lution gradient. Perch caught in this polluted area exhibited abnormalities in several vital physiological processes, as described below. Except for gonad size, no sex differences in the various physiological parameters were seen. Therefore, male and female fish have been treated as one group for all parameters except GSI.

### Liver and Gonad Size

On all sampling occasions, liver size, as estimated by the LSI, was significantly greater in fish caught up to 8 km from the plant than in corresponding controls (Fig. 2). However, in September 1985 the LSI of fish caught at the sampling site 10 km from the mill outlet was not significantly different from that of the control fish, indicating that between 8 and 10 km, levels of BKME decreased below the threshold of this response. Liver hypertrophy has also been found in fourhorn sculpin (*Myoxocephalus quadricornis*) exposed to BKME in the laboratory (Andersson et al. 1987). The specific factors responsible for the liver enlargement have yet to be determined, although hypertrophy is probably associated with the pollution-induced metabolic disturbances and/or enhanced activity of xenobiotic biotransformation enzymes described below. For example, the enlargement might be the result of an increase in hepatic fat storage and/or stimulated protein synthesis in the endoplasmic reticulum of the liver cells (Klaunig et al. 1976).

Gonad size, expressed as GSI, was smaller in both male and female fish caught near the mill outlet compared with control fish (Fig. 3). Moreover, the effect showed a graded response along the pollution gradient. In September 1985, even the perch caught 10 km from the plant had smaller gonads than the control fish. The results from May 1984, the spawning time for perch, are omitted from the figure, since gonads are weighed with great uncertainty at this time. Still, these results showed a similar trend.

The decreased gonad size in fish inhabiting the vicinity of the pulp mill indicates that gonadal development in these fish was inhibited. Consequently, this effect may disturb normal reproduction in the area, since gonads are responsible both for producing the hormones necessary for sexual maturation and for the development of the germ cells. Such a suggestion is supported by signs of reduced reproduction of perch (inhibited gonad growth and low abundance of perch fry) observed in the most polluted part of the receiving water (Neuman and Karås 1988; Sandström et al. 1988). The toxic actions of pollutions may affect the gonadal development at various levels. Normal development of the reproductive organs depends on critical molecular and cellular processes regulated by hormones and other factors (Bentley 1982) which could be disrupted or influenced by the physicochemical environment.

### Carbohydrate Metabolism and Hepatic Ascorbic Acid Content

In an earlier laboratory study, fourhorn sculpin exposed to BKME exhibited increased levels of muscle glycogen (Andersson et al. 1987). In the present field study on perch inhabiting the BKME-polluted area, a similar elevation in muscle glycogen content was evident on the first three sampling occasions (Table 1). Liver glycogen levels also appeared to increase in perch from the polluted area (Fig. 4). In addition, compared with the control perch, these fish showed decreased blood glucose levels in May and August 1984, no marked difference in October 1984, and significantly increased levels in September 1985 (Table 1). Blood lactate levels increased as distance to the mill outlet decreased in May 1984, October 1984, and September 1985. In August 1984, there was no apparent relationship

TABLE 1. Levels of blood glucose, blood lactate, muscle glycogen, liver UDP glucuronosyltransferase activity (UDP GT), and plasma bilirubin in perch from the control site (Ref) and from sites polluted with BKME. Polluted sampling sites are situated 10, 8, 4.5, and 2 km from the outlet. The results are expressed as mean ± SD (number of fish in parentheses). Differences between samples from the control site (or at 8 km in October 1984) and fish from the polluted sites were statistically significant at the $*p < 0.05$ and $*p < 0.01$ level, respectively.

| Sampling site | Blood glucose (mg/100 mL) | Blood lactate (mg/100 mL) | Muscle glycogen (mg/g) | UDP GT (nmol·mg protein$^{-1}$·min$^{-1}$) | Plasma bilirubin (μM) |
|---|---|---|---|---|---|
| May 1984 | | | | | |
| Ref. | 67.2±22.7 (20) | 13.7±7.8 (24) | 0.43±0.35 (21) | 0.13±0.07 (23) | 1.93±1.16 (24) |
| 8 km | 78.5±13.0* (21) | 29.3±10.6**(28) | 0.96±0.59**(26) | 0.24±0.09**(26) | 1.11±0.64 (28) |
| 4.5 km | 50.5±16.1**(27) | 33.1±13.0**(27) | 1.02±0.56**(24) | 0.37±0.12**(26) | 4.75±2.48**(18) |
| 2 km | 45.2±13.8**(27) | 50.1±10.1**(27) | 0.71±0.51 (25) | 0.37±0.13**(28) | 6.92±4.89** (7) |
| August 1984 | | | | | |
| Ref. | 69.4±24.6 (21) | 18.8±9.9 (21) | 0.21±0.18 (22) | 0.13±0.09 (19) | 1.50±2.12 (21) |
| 8 km | 60.6±18.4 (23) | 17.9±4.8 (20) | 0.28±0.18 (24) | 0.15±0.13 (22) | 4.16±2.36**(21) |
| 4.5 km | 51.7±9.8** (24) | 10.2±9.6** (24) | 0.45±0.19**(23) | 0.17±0.12 (23) | 3.16±2.05* (22) |
| 2 km | 57.8±18.0 (16) | 15.9±10.7 (13) | 0.46±0.32**(15) | 0.17±0.10 (13) | 5.10±1.53**(13) |
| October 1984 | | | | | |
| 8 km | 78.2±25.2 (10) | 16.5±11.9 (16) | 0.43±0.33 (17) | 0.23±0.17 (17) | 2.24±1.89 (15) |
| 4.5 km | 90.1±14.0 (10) | 22.6±8.6 (21) | 0.86±0.53* (21) | 0.34±0.09* (21) | 1.22±1.38 (17) |
| 2 km | 83.0±15.5 (15) | 41.4±22.9**(22) | 1.00±0.82**(22) | 0.34±0.14* (22) | 1.85±1.48 (18) |
| September 1985 | | | | | |
| Ref. | 52.2±9.6 (25) | 7.6±2.7 (23) | 0.30±0.18 (17) | 0.14±0.09 (22) | 1.91±1.55 (25) |
| 10 km | 62.4±12.5* (23) | 6.5±1.3 (24) | 0.34±0.24 (21) | 0.16±0.11 (23) | 1.30±1.01 (23) |
| 8 km | 67.5±14.7**(12) | 12.1±3.3* (11) | 0.41±0.25 (10) | 0.28±0.20**(13) | 0.52±0.58**(10) |
| 4.5 km | 82.4±18.2**(22) | 8.7±3.5 (20) | 0.25±0.10 (20) | 0.17±0.10 (22) | 0.91±0.57* (19) |
| 2 km | 82.1±12.4**(24) | 13.8±5.8** (25) | 0.36±0.16 (23) | 0.16±0.11 (24) | 2.74±1.41* (24) |

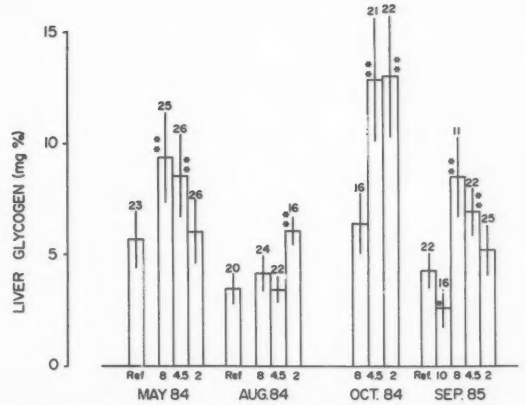


FIG. 4. Liver glycogen levels in perch from the control site and sites polluted with BKME. See legend to Fig. 2 for further explanations.

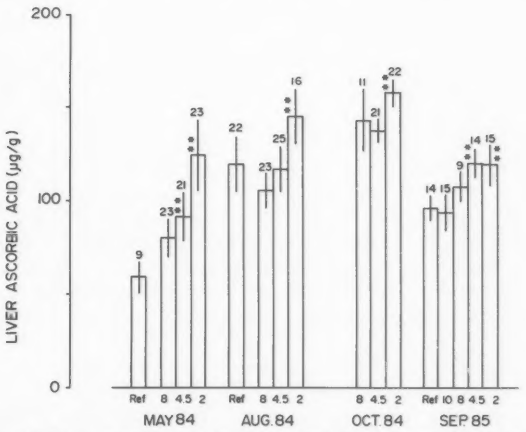


FIG. 5. Liver ascorbic acid levels in perch from the control site and sites polluted with BKME. See legend to Fig. 2 for further explanations.

between lactate levels and distance from the pollution source (Table 1).

Previous studies on fish have generally found that exposure to pulp mill effluents decrease liver and muscle glycogen levels while increasing blood glucose and lactate levels (McLeay and Brown 1974, 1975). Such effects are typical secondary stress responses induced by the increased release of hormones from the pituitary, the interrenals, and the chromaffin tissue (Mazeaud and Mazeaud 1981). The results obtained in the present study, however, are not typical of stress responses. Instead, it appears as if compounds in the BKME have a complex and severe influence on carbohydrate metabolism. The elevated glycogen content in liver and muscle tissue, the mostly increased blood lactate levels, and the varying levels of blood glucose are all indicative of abnormal carbohydrate metabolism in the affected fish, possibly the result of impaired hormonal control. Such a metabolic imbalance must be regarded as serious and probably affects the energy balance, especially during periods of enhanced energy demand, e.g. during sexual maturation and in stress and starvation situations.

Ascorbic acid is an essential dietary vitamin in fish, although its precise role remains unclear (Thomas et al. 1985). In previous studies, stress and environmental contaminants, such as toxaphene and cadmium, caused depletion of ascorbic acid reserves in fish (Thomas et al. 1982). Mammals deficient in ascorbic acid show decreased rates of collagen formation and reduced activity of several xenobiotic metabolizing enzymes including cytochrome P-450 monooxygenase and UDP glucuronosyltransferase (Zannoni and Sato 1975; Paterson and Fry 1983). The present study indicates that the liver reserves of ascorbic acid in perch exposed to BKME are elevated in a dose-dependent manner (Fig. 5). Such an increase may be due to increased synthesis reflecting an enhanced demand for ascorbic acid in processes such as detoxification or wound healing (Thomas et al. 1982) in the BKME-exposed fish.

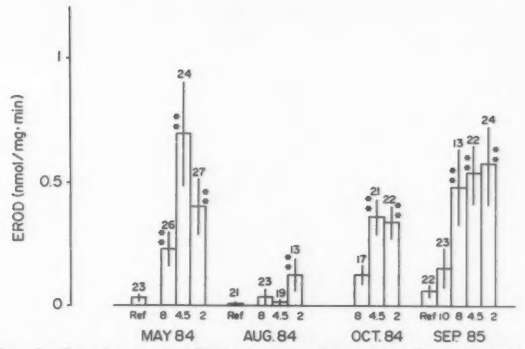


FIG. 6. Cytochrome P-450-dependent ethoxyresorufin-*O*-deethylase (EROD) activities in liver microsomes of perch from the control site and sites polluted with BKME. See legend to Fig. 2 for further explanations.

## Xenobiotic Biotransformation Enzymes and Plasma Bilirubin

The cytochrome P-450 enzyme system plays a central role in the metabolism of many lipophilic environmental pollutants. Through the action of cytochrome P-450, functional groups are introduced into the substrate molecule, which facilitate further metabolism by conjugating enzymes such as UDP glucuronosyltransferase. Induction of the cytochrome P-450-dependent activities by many xenobiotics, such as polyaromatic hydrocarbons (PAH), is one of this system's major characteristics (Stegeman 1981). Induction of cytochrome P-450-dependent deethylation of ethoxyresorufin by petroleum contaminants in fish is a very sensitive biological response that should be useful in biological monitoring programs (Payne et al. 1987).

Fish in the vicinity of the pulp mill showed markedly induced cytochrome P-450- dependent 7-ethoxyresorufin-*O*-deethylase activities on all sampling occasions (Fig. 6). In September 1985, when the investigation area was extended to include a sampling site 10 km from the discharge point, fish also exhibited induced cytochrome P-450 activities at this distance, although the induction was not as pronounced as it was in fish from the innermost localities.

The hepatic UDP glucuronosyltransferase activity towards *p*-nitrophenol in fish from sampling sites near the pulp mill in May 1984 was higher than that in control fish (Table 1). Likewise, fish from the two innermost localities in October 1984 showed higher levels of hepatic UDP glucuronosyltransferase activity than fish from the sampling site 8 km from the outlet. In August 1984 and September 1985, the effects on UDP glucuronosyltransferase were absent or less pronounced (Table 2).

TABLE 2. Inorganic ions in blood plasma of perch from the control site (Ref) and from sites polluted with BKME. See Table 1 for further explanations.

| Sampling site | Plasma sodium (mM) | Plasma potassium (mM) | Plasma magnesium (mM) | Plasma calcium (mM) |
|---|---|---|---|---|
| May 1984 | | | | |
| Ref. | 149±8 (23) | 3.3±0.6 (21) | 0.75±0.10 (16) | 2.98±0.80 (16) |
| 8 | 148±8 (28) | 3.1±0.4 (27) | 0.97±0.30**(15) | 2.83±1.04 (15) |
| 4.5 | 148±5 (27) | 3.6±0.5 (25) | 0.79±0.15 (24) | 2.45±0.69 (24) |
| 2 | 150±8 (20) | 3.7±0.5* (19) | 0.90±0.16* (19) | 2.66±0.73 (19) |
| August 1984 | | | | |
| Ref. | 154±11 (20) | 3.9±0.5 (19) | 0.94±0.14 (21) | 2.81±0.25 (21) |
| 8 | 150±8 (21) | 4.4±0.7* (20) | 0.64±0.08**(22) | 2.09±0.17**(22) |
| 4.5 | 154±15 (22) | 4.0±0.3 (19) | 0.64±0.12**(23) | 2.03±0.19**(23) |
| 2 | 152±7 (16) | 3.8±0.6 (12) | 0.70±0.10**(16) | 2.18±0.27**(16) |
| October 1984 | | | | |
| 8 | 147±8 (16) | 2.9±0.8 (16) | 0.94±0.30 (15) | 2.60±0.59 (15) |
| 4.5 | 147±9 (20) | 3.1±0.6 (20) | 0.91±0.10 (19) | 2.58±0.70 (19) |
| 2 | 152±9 (22) | 3.3±0.6 (21) | 0.79±0.09* (21) | 2.12±0.49* (21) |
| September 1985 | | | | |
| Ref | 143±7 (25) | 3.0±0.5 (25) | 0.99±0.26 (25) | 2.38±0.33 (25) |
| 10 | 136±5**(22) | 2.6±0.4**(24) | 1.61±0.34**(20) | 2.21±0.32 (24) |
| 8 | 140±7 (11) | 3.1±0.6 (13) | 1.22±0.24* (13) | 2.50±0.32 (13) |
| 4.5 | 141±7 (22) | 3.2±0.5 (22) | 1.33±0.31**(22) | 2.27±0.26 (22) |
| 2 | 142±6 (25) | 3.3±0.4 (25) | 1.23±0.19**(25) | 2.72±0.53**(24) |

In the laboratory study on fourhorn sculpin, cytochrome P-450-dependent activities were induced by BKME from a pine pulp line whereas UDP glucuronosyltransferase activity was not affected by BKME (Andersson et al. 1987). The results from this field study and the previous laboratory study strongly suggest the presence of potent cytochrome P-450 inducers, such as aromatic chlorinated compounds, in BKME. In fact, well-known cytochrome P-450 inducers such as dibenzodioxins and dibenzofurans have been detected in muscle of perch from the polluted area (Södergren et al. 1988).

The toxicological and physiological significance of increased cytochrome P-450 and UDP glucuronosyltransferase activites has been subject to much debate. An enhanced rate of xenobiotic biotransformation is often associated with a faster termination of toxic actions. However, induction of cytochrome P-450 activities can also result in the formation of electrophilic metabolites and chemical carcinogens, which are biologically more active than the parent compound and probably responsible for the acute or chronic toxicity associated with certain xenobiotics (Ingelman-Sundberg 1980). Induction of cytochrome P-450 and UDP glucuronosyltransferase activities can also lead to an increase in the metabolism of endogenous compounds, such as steroids. Thus, reduced steroid levels and a subsequent impairment of reproductive functions can result from such an induction. Biliary excretion of estradiol in rainbow trout (*Salmo gairdneri*) was found to increase twofold after exposure to β-naphthoflavone, a PAH-type inducer (Förlin and Haux 1985). Furthermore, Sivarajah et al. (1978) reported reduced levels of plasma steroids after PCB treatment. Both reports describe elevated hepatic cytochrome P-450 and UDP glucuronosyltransferase activities concomitant with altered steroid levels. Extensive investigations on a fish population in San Francisco Bay have recently begun to reveal that a correlation exists between organic contaminants, cytochrome P-450 induction, and reproductive success (Spies et al. 1985).

In May and August 1984, the levels of total bilirubin in blood plasma from fish caught nearby the pulp mill were significantly higher than levels measured in control fish (Table 1). However, no effects were detected on the bilirubin levels in October 1984 whereas in September 1985, plasma bilirubin was lower in fish from the less polluted sites than in control fish and significantly higher in fish from the site 2 km from the plant. Bilirubin is a major end-product of hemoglobin degradation. Changed plasma bilirubin levels may therefore reflect disturbed functions on many levels, e.g. destruction of RBC, Hb catabolism, obstruction of bile ducts, and damage to liver cells. An increased breakdown of RBC in vitro by dehydroabietic acid, a component in BKME, was observed by Bushnell et al. (1985).

As bilirubin is excreted into the bile predominantly in the form of glucuronide conjugates, elevated bilirubin levels in blood have been suggested to be the result of inhibition of the conjugating enzyme UDP glucuronosyltransferase (measured by using *p*-nitrophenol as substrate) (Oikari and Nakari 1982). Elevated levels of bilirubin in blood plasma of sockeye salmon (*Oncorhynchus nerka*) exposed to a sublethal dose of dehydroabietic acid were found to be mainly in the conjugated form, indicating no problems with the conjugation step in the liver (Kruzynski 1979). Furthermore, in the present field study the UDP glucuronosyltransferase activity towards *p*-nitrophenol was elevated or remained unaffected by BKME. However, in mammals, UDP glucuronosyltransferases are a family of isoenzymes in which glucuronidation of *p*-nitrophenol and bilirubin is catalyzed by two distinct entities (Burchell and Blanckaert 1984). Thus the hepatic UDP glucuronosyltransferase activity towards *p*-nitrophenol in fish may not be related to bilirubin excretion.

### Ion Balance

No substantial differences were detected between sodium ion concentration in blood plasma of control perch and concentra-

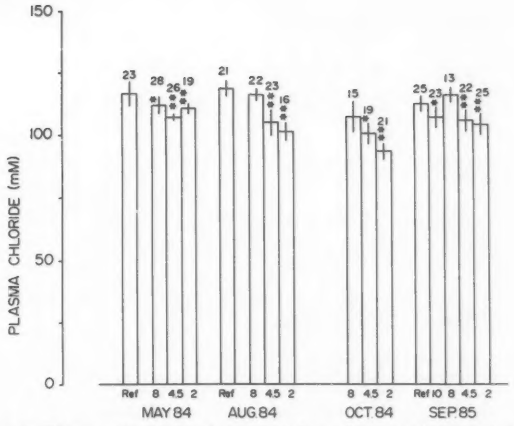


FIG. 7. Plasma chloride levels in perch from the control site and sites polluted with BKME. See legend to Fig. 2 for further explanations.

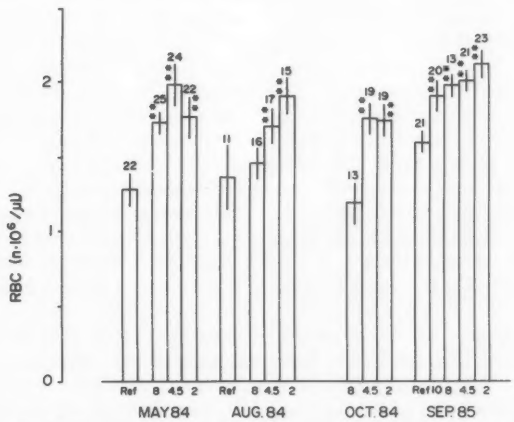


FIG. 8. RBC count in perch from the control site and sites polluted with BKME. See legend to Fig. 2 for further explanations.

tions in perch from the polluted capture sites on any sampling occasion (Table 2). The other measured plasma ions were, however, affected to various extents. Thus, plasma potassium levels in perch seemed to increase slightly as distance to the plant decreased in May 1984, October 1984, and September 1985 (Table 2) whereas plasma chloride levels showed an obvious successive decrease in the same pollution gradient on all sampling occasions (Fig. 7). Levels of the divalent ions calcium and magnesium showed variable responses to the effluent pollutants. In May 1984, perch from the polluted sites had significantly higher plasma magnesium but unaffected plasma calcium levels (Table 2). In August 1984, the levels of both these ions were significantly reduced in perch from all polluted sites whereas in October 1984, only the fish from the most polluted site showed decreased values. At the last sampling event (September 1985), magnesium levels were higher in all exposed fish groups, while the calcium level was only higher in perch captured nearest the plant (Table 2).

Taken together, the observed alterations in blood plasma content of the various inorganic ions indicate that fish, living in the polluted areas around the kraft pulp mill plant, suffer from impaired ion regulation. The observed hyperkalemic response may have been caused by leakage of intracellular potassium through damaged cell membranes into the surrounding body fluids. Obviously, ion regulatory mechanisms are incapable of compensating for this alteration. A similar effect on potassium balance has previously been shown in fish exposed to inorganic lead both in the field and in the laboratory (Larsson et al. 1985).

A decreased plasma chloride level has earlier been observed in fourhorn sculpin exposed for 9 mo to BKME in a laboratory experiment (Härdig et al. 1988). In contrast, no effects on ion balance were found in fish exposed for 200 d to BKME (McLeay and Brown 1979). The reduced plasma chloride content in the present field study suggests that BKME exposure disturbs gill function, thereby impairing active uptake of chloride ions via the gills and/or causing chloride ions to leak from the extracellular fluids into the surrounding hypotonic water as a result of pathological changes in the gill membranes. Similar effects on plasma chloride levels have been observed both in fish living in metal-polluted waters (Larsson et al. 1985) and in laboratory experiments with fish exposed to various environmental pollutants, including metals (Larsson et al. 1985) and chlorinated hydrocarbons (Förlin et al. 1979; Haux and Larsson 1979). Thus, chloride regulation in fish appears to be highly sensitive to environmental pollutants. Impaired chloride regulation can have serious consequences, since the ability of fish to maintain chloride homeostasis is of crucial importance to all fish species living in coastal waters of varying salinity and in species such as perch that migrate between brackish water and freshwater (Eddy 1981; Larsson et al. 1985). It has also been shown that fish suffering from a disturbed ion balance (e.g. reduced chloride level in blood plasma) are less able to withstand additional stress (Larsson et al. 1984).

The observed disturbances in the regulation of plasma potassium, calcium, and magnesium can also be regarded as serious, since these ions are involved in several vital functions and processes. For example, membrane processes and nerve and muscle functions are very sensitive to alterations both in the total osmotic concentration and in the relative ion composition of the body fluids. Thus, disturbances in osmotic and ionic regulation leading to abnormal levels of inorganic ions in various body tissues can result in severe strains on and disturbances in many physiological functions, e.g. impaired motor functions and muscle coordination, nerve disturbances, and behavioural changes (Larsson et al. 1985).

## Hematology

The number of RBC was strongly elevated in perch living near the pulp plant (Fig. 8). A 15–50% increase was apparent on all four sampling occasions. Furthermore, in August 1984 and September 1985, the RBC count in perch showed a distinct graded response along the pollution gradient. The elevated RBC count was also accompanied by significant increases in Ht and Hb content (Table 3). However, the increases in Ht value and Hb content were not as pronounced as the increase in RBC count which led to significantly lowered values for mean cell Hb content (MCH) and mean cellular volume (MCV) in perch in the vicinity of the pulp plant (Table 3).

Taken together, these alterations in RBC status show that perch in the BKME-polluted water areas had successively higher numbers of small erythrocytes with low Hb content along the pollution gradient towards the pulp plant. Since young erythrocytes are usually smaller and contain less Hb than older ones, we interpret this response as indicating that production

TABLE 3. Hematological parameters in fish from the control site (Ref) and from sites polluted with BKME. Se

| Sampling site | ALA-D (μg/mLXh) | Ht (%) | Hb (mmol/L) | metHb (%) | MCH (fmol/cell) |
|---|---|---|---|---|---|
| May 1984 | | | | | |
| Ref. | 54±24 (10) | 26.7±4.6 (22) | 0.88±0.14 (22) | 1.3±0.4 (22) | 0.69±0.13 (2 |
| 8 km | 84±20**(23) | 30.9±2.7**(25) | 0.89±0.11 (25) | 1.9±0.6**(25) | 0.52±0.06**(2 |
| 4.5 km | 71±15* (23) | 29.7±2.5**(24) | 0.99±0.10* (24) | 1.3±0.4 (23) | 0.50±0.06**(2 |
| 2 km | 62±22 (18) | 30.6±3.6**(22) | 1.01±0.23**(22) | 2.0±0.8**(22) | 0.58±0.11**(2 |
| August 1984 | | | | | |
| Ref. | 76±23 (21) | 29.9±4.2 (13) | 1.04±0.27 (13) | n.d. | 0.79±0.22 (1 |
| 8 km | 91±25* (22) | 29.9±2.4 (16) | 0.96±0.11 (16) | n.d. | 0.68±0.16**(1 |
| 4.5 km | 87±21 (24) | 28.5±2.8 (17) | 1.09±0.13 (17) | n.d. | 0.64±0.07**(1 |
| 2 km | 96±24* (16) | 34.9±4.2**(15) | 1.16±0.15 (15) | n.d. | 0.61±0.06**(1 |
| October 1984 | | | | | |
| 8 km | 105±25 (17) | 26.5±3.4 (13) | 0.92±0.13 (13) | 1.8±0.8 (13) | 0.80±0.18 (1 |
| 4.5 km | 100±23 (21) | 28.2±2.4 (19) | 1.05±0.13* (18) | 4.8±1.8**(18) | 0.61±0.08**(1 |
| 2 km | 82±20**(22) | 29.3±2.8* (19) | 1.01±0.16 (19) | 3.7±1.3**(19) | 0.58±0.04**(1 |
| September 1985 | | | | | |
| Ref. | 68±22 (20) | 27.6±2.6 (23) | 0.65±0.07 (21) | 1.0±0.2 (24) | 0.41±0.03 (2 |
| 8 km | 87±17* (20) | 29.0±2.5 (24) | 0.73±0.06**(20) | 1.0±0.3 (24) | 0.38±0.03 (2 |
| 10 km | 93±16**(12) | 30.3±2.8* (13) | 0.72±0.05**(13) | 1.3±0.4* (13) | 0.37±0.03* (1 |
| 4.5 km | 105±20**(14) | 30.0±1.7* (22) | 0.68±0.05 (21) | 0.9±0.2 (22) | 0.34±0.02**(2 |
| 2 km | 104±24**(20) | 33.1±2.3**(25) | 0.75±0.07**(23) | 1.2±0.3* (25) | 0.36±0.03**(2 |

ME. See Table 1 for further explanations.

| MCH (fmol/cell) | MCV (pL) | Thrombocytes/ (μL × $10^4$) | Granulocytes/ (μL × $10^4$) | WBC (μL × $10^4$) |
|---|---|---|---|---|
| 0.13 (22) | 19.0±3.2 (22) | 20±8 (22) | 14±6 (22) | 137±70 (22) |
| 0.06**(25) | 16.0±1.1**(25) | 35±14**(25) | 32±17**(25) | 212±76**(25) |
| 0.06**(24) | 13.7±2.1**(24) | 29±12* (24) | 34±13**(24) | 212±89**(24) |
| 0.11**(22) | 16.5±2.5**(22) | 22±14 (25) | 27±12**(25) | 116±58 (25) |
| 0.22 (11) | 20.5±5.3 (11) | 43±29 (12) | 25±13 (12) | 160±60 (12) |
| 0.16**(16) | 18.7±3.5 (16) | 36±16 (16) | 30±12 (16) | 162±56 (16) |
| 0.07**(17) | 15.2±1.6**(17) | 46±15 (17) | 32±15 (17) | 182±42 (17) |
| 0.06**(15) | 16.8±1.5**(15) | 39±20 (15) | 26±11 (15) | 131±53 (15) |
| 0.18 (13) | 21.2±4.9 (13) | 19±9 (12) | 18±11 (12) | 100±31 (12) |
| 0.08**(18) | 15.4±1.1**(19) | 26±14 (19) | 21±13 (19) | 87±21 (19) |
| 0.04**(19) | 16.3±2.5**(19) | 32±14* (19) | 22±11 (19) | 87±28 (19) |
| 0.03 (21) | 15.7±1.2 (21) | 35±16 (21) | 18±6 (21) | 135±36 (21) |
| 0.03 (20) | 14.7±0.9 (19) | 34±18 (20) | 22±11 (20) | 109±41* (20) |
| 0.03* (13) | 14.7±1.2 (13) | 25±14 (13) | 22±10 (13) | 99±49* (13) |
| 0.02**(21) | 14.5±0.9* (21) | 22±11* (21) | 24±11* (21) | 76±33**(21) |
| 0.03**(23) | 15.3±1.1 (23) | 18±12**(23) | 16±4 (23) | 58±20**(23) |

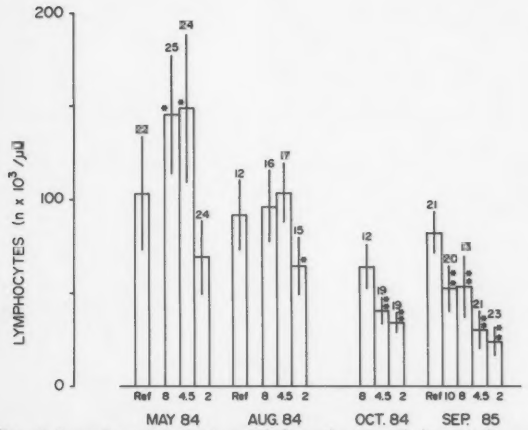


FIG. 9. Lymphocyte count in perch from the control site and sites polluted with BKME. See legend to Fig. 2 for further explanations.

of RBC was stimulated in the BKME-exposed perch. The increased activity of the enzyme ALA-D (involved in Hb synthesis) in perch from the BKME-polluted waters (Table 3) also suggests that erythrocyte production was stimulated.

Increased levels of immature RBC, indicating stimulated erythropoiesis, have previously been observed in BKME-exposed fish (Howard et al. 1971; McLeay 1973). Furthermore, rainbow trout caged for 10 d in the receiving water body of a pulp mill exhibited elevated Ht value and Hb content (Tana and Nikunen 1986). In contrast, previous laboratory studies (Härdig et al. 1988) have shown the induction of a slight anemic response (lowered RBC count, Ht value, and Hb content) in fourhorn sculpin exposed for 5–9 mo to BKME. Thus, RBC status seems to be sensitive to BKME, although the ultimate response may differ, depending on factors such as the quality of the effluents, the duration and grade of exposure, the fish species, and the ionic composition of the receiving water.

In fish, the production of RBC is stimulated by an insufficient supply of oxygen to the tissues. As there were no signs of reduction in dissolved oxygen content in the water, the increased RBC count found in the present field investigation in perch from BKME-polluted water areas may be a result of an impaired binding of oxygen to the Hb, gill damage leading to gas diffusion problems across the gills, or increased demand for oxygen by certain tissues. Although Fujiya (1964) and Lehtinen et al. (1984) reported that BKME caused gill damage in fish, morphometric studies on gills from perch caught in September 1985 at the two innermost sampling sites did not reveal any structural changes which could explain the effects on the erythrocyte status (Härdig et al. 1988; and unpubl. data). Thus the most plausible explanation for stimulated erythropoiesis is an increased demand for oxygen by certain tissues. Such a hypothesis is supported by the severe metabolic imbalance, which in turn influences the energy balance, and by the strongly increased detoxification activity in the liver of the BKME-exposed fish, both of which could contribute to an increased need for oxygen by the tissues.

The methemoglobin levels (Table 3) showed a slight elevation in the blood of most perch caught in May 1984 and September 1985 at the sampling sites near the pulp plant. In October 1984, methemoglobin levels were more than doubled in perch from the two innermost sampling sites compared with perch from the more remote site (8 km). These results agree well with the findings on fourhorn sculpin exposed for 5–9 mo to BKME in the laboratory (Härdig et al. 1988). Thus, BKME apparently includes substances with the ability to increase the rate of oxidation of Hb to methemoglobin and/or inhibit the reducing systems responsible for the reverse reaction. Although the elevated proportion of methemoglobin (<4–5%) in the affected perch was too low to appreciably reduce the oxygen transport capacity of the blood, it may be indicative of a more profound interaction between toxic substances in the BKME and mechanisms vital to the proper maintenance of RBC.

WBC counts were highly abnormal in perch living in the vicinity of the pulp plant (Table 3; Fig. 9). Most striking was the marked reduction of the lymphocyte count in perch from the most polluted sites (Fig. 9). In October 1984 and September 1985, this decrease in lymphocyte count followed a distinct dose–response pattern. On the last sampling occasion, even the perch from the most remote site (10 km) exhibited a significantly reduced number of lymphocytes. On the whole, the total number of WBC (Table 3) varied along with the number of lymphocytes, the dominant type of WBC. The number of neutrophilic granulocytes was significantly higher in BKME-exposed fish in May 1984. Otherwise, this type of WBC did not show any major alterations in the exposed perch. The response of the thrombocyte count varied with sampling occasion. Hence, perch from the polluted receiving body of water showed higher numbers of thrombocytes in May and October 1984, no significant alterations in August 1984, and a dose-dependent reduction in September 1985.

A depressed total WBC count, indicating acute stress, was observed in fish exposed to BKME in short-term laboratory investigations (McLeay 1975; McLeay and Gordon 1977). In contrast, long-term exposure of fish to BKME in the laboratory did not signifcantly affect the total WBC count (McLeay and Brown 1974) or the number of lymphocytes, neutrophilic granulocytes, or thrombocytes (Härdig et al. 1988).

A reduced lymphocyte count is a common secondary stress response in fish subjected to acute stress (Ellis 1981; Larsson et al. 1985). However, the chronic exposure of the fish to BKME-polluted waters near a pulp plant cannot be considered an acute stress situation. Instead, the lymphocyte response apparently reflects a more profound disturbance in lymphocyte production and would be expected to impair the functioning of the immune system. Such disturbances might severely reduce the health and survival of fish by decreasing their ability to withstand infections or other stresses (Ellis 1981; Wedemeyer and McLeay 1981).

The high frequency of external pathological fin alterations observed on all sampling occasions, but especially in May 1984, in the perch living in the most polluted areas might have been a direct consequence of the impairment of their immune system. These effects were successively more frequent as the distance to the pulp mill decreased (Lindesjöö and Thulin 1987). The most typical symptom was an eroded and shortened tail fin, probably the result of a fin rot infection.

The increased counts of lymphocyte, neutrophilic granulocytes, and thrombocytes (Fig. 9: Table 3) in perch caught in May 1984 at the most remote polluted sites (4.5 and 8 km) may have been a natural response to the acute tail fin infection. Such normal immune responses to infections were not evident or were much weaker in the more infected perch from the most polluted site, which further suggested an impaired immunological defense system in these highly exposed fish.

## General Conclusions

The present health investigation clearly indicates that physiological variables of perch living in the receiving water body of a pulp bleaching plant are strongly affected by the pollutants. Apparently, bleaching effluents can cause extensive physiological responses, such as reduced gonad growth, liver enlargement, strong induction of cytochrome P-450-dependent enzyme activities (increased by 5–20 times), metabolic imbalance, disturbed ion balance, stimulated RBC production, and a weakened immune system. This extensive symptom picture is in good agreement with effects previously observed in fish exposed to effluents from the Norrsundet pulp mill in the laboratory (Andersson et al. 1987; Härdig et al. 1988). This supports the conclusion that the physiological responses seen in this field study are caused by BKME. Moreover, abnormalities in many of the physiological parameters (LSI, GSI, liver glycogen, 7-ethoxyresorufin-*O*-deethylase activity, liver ascorbic acid, plasma chloride, MCH, RBC, Ht, and lymphocytes) were found in BKME-exposed fish at all four sampling times, which further supports the symptom picture. Thus, the BKME discharge causes serious disturbances in vital physiological functions which certainly affect the health status and survival of the fish.

The disturbances were most pronounced at the two innermost capture sites (2 and 4.5 km; degree of waste water dilution: 166 and 330 times, respectively), but many physiological effects (e.g. cytochrome P-450 induction, reduced gonad growth, and altered RBC and WBC characteristics) were also detected in perch caught 8 km from the pulp bleaching plant (degree of waste water dilution: 1140 times). In addition, in September 1985, some of these effects were even detected in perch from the remote 10-km site. This indicates that BKME affects organisms over a distance of at least 8–10 km from the discharge point, where the waste water has been diluted over 1100 times. Thus, the area over which the waste water exerts serious biological effects seems to be much larger than previously believed (Anonymous 1985).

These physiological disturbances, prevailing in fish caught over a broad area, may have serious effects on the fish community. In fact, parallel investigations in the same area have shown an altered fish species composition, markedly reduced fry numbers, and an increased fish growth (Neuman and Karås 1988; Sandström et al. 1988). Extrapolation of the physiological effects seen in this study on the individual level to responses of the fish population will be discussed elsewhere (O. Sandström, Swedish Environmental Protection Board, Marine Section, Box 584, S-740 71 Öregrund, Sweden, pers. comm.).

In relative terms, cytochrome P-450-dependent 7-ethoxyresorufin-*O*-deethylase activity was the physiological variable most affected in perch exposed to BKME in this study (Fig. 10). As also illustrated in Fig. 10, many of the physiological variables exhibited a graded response along the pollution gradient. This indicates that the level of physiological disturbance was dose dependent, further strengthening the cause/effect relationship. These facts, together with the good consistency in the physiological responses over time, mean that many variables (especially LSI, GSI, liver glycogen levels, liver ascorbic acid

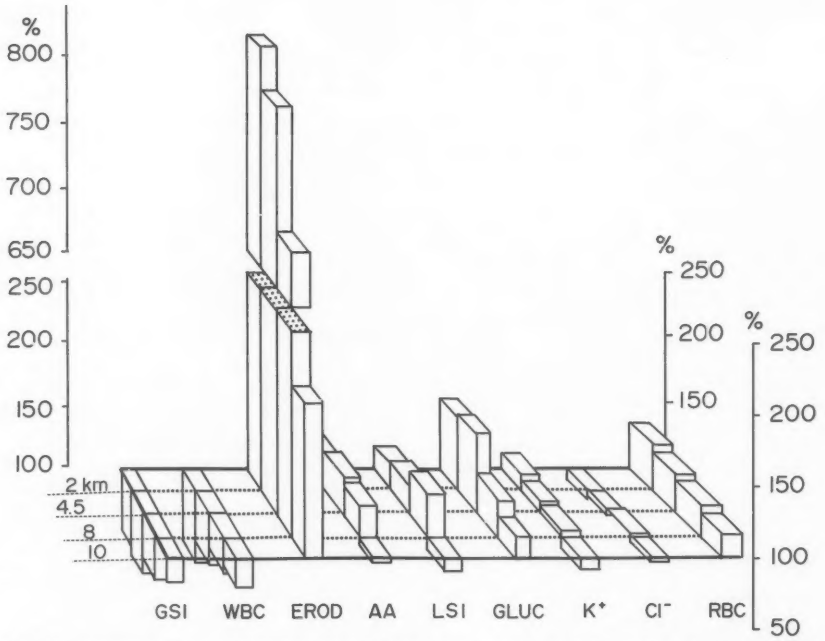


FIG. 10. Overview of some physiological and biochemical changes in fish caught in September 1985 at polluted sites situated 10, 8, 4.5, and 2 km from the outlet. The values are expressed as a percentage of the values in fish from the control site which is set at 100%. GSI, gonad somatic index; LSI, liver somatic index; GLUC, blood glucose; AA, ascorbic acid in liver; EROD, liver microsomal cytochrome P-450-dependent ethoxyresorufin-*O*-deethylase activity; $K^+$, plasma potassium; $Cl^-$, plasma chloride, RBC, red blood cell count; WBC, total white blood cell count.

content, cytochrome P-450-dependent ethoxyresorufin-*O*-deethylase activity, plasma chloride content, RBC, and lymphocyte count) should be useful as reliable indicators for detecting sublethal toxic effects on natural populations of fish and should delineate areas of potential impact of environmental pollutants.

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# Life History Variation of Polymorphic Arctic Charr (*Salvelinus alpinus*) in Thingvallavatn, Iceland[1]

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The Arctic charr (*Salvelinus alpinus*) in Thingvallavatn, Iceland, exhibits four morphs. Small benthivorous charr grow slowly, have an asymptotic length of 13 cm, and mature at an age of 2–4 yr. Large benthivorous charr grow faster and almost rectilinearly with age. They mature at an age of 3–11 yr. Planktivorous and piscivorous charr have even higher initial growth rate, but planktivorous charr cease growing at 20 cm and piscivorous charr at a fork length of 30 cm. They mature at 3–5 yr and 5–10 yr of age, respectively. The gonadosomatic index and the annual allocation of energy into gonads relative to soma were higher for planktivorous and piscivorous than for benthivorous charr. The results indicate that piscivorous and planktivorous charr belong to the same gene pool; piscivorous charr are probably recruited from the most fast-growing planktivorous individuals and start feeding on fish at a length of 23 cm. The benthivorous morphs may belong to different stocks due to significant differences in several life history variables as well as genetics, spawning time, and feeding habitat. The four morphs seem to be differentiated within the lake through trophic radiation, but should be considered as conspecifics.

Les ombles arctiques (*Salvelinus alpinus*) du Thingvallavatn (Icelande) présentent quatre types morphologiques. Les petits ombles benthivores ont une croissance lente, atteignent une longueur asymptotique de 13 cm, et maturent quand ils ont entre 2 et 4 a. Les gros ombles benthivores croissent plus rapidement et de façon presque rectiligne en fonction de l'âge. Ils atteignent la maturité à un âge se situant entre 3 et 11 a. Les ombles planctivores et piscivores présentent un taux de croissance initial encore plus élevé, mais les individus planctivores cessent de croître quand ils ont atteint une longueur à la fourche de 20 cm et les individus piscivores à une longueur de 30 cm. Les gammes d'âges de maturation sont, respectivement, de 3 à 5 a et de 5 à 10 a. L'indice gonadosomatique et le rapport annuel de l'énergie répartie entre les gonades et le soma étaient plus élevés chez les ombles planctivores et piscivores que chez les ombles benthivores. Les résultats obtenus indiquent que les ombles piscivores et planctivores appartiennent au même réservoir génique, les individus piscivores provenant probablement des individus planctivores à croissance la plus rapide et commençant à se nourrir de poissons lorsqu'ils atteignent 23 cm de longueur. Les types benthivores pourraient appartenir à d'autres stocks car divers paramètres de leur cycle vital de même que leurs caractéristiques génétiques, leur période de frai et leur habitat d'alimentation diffèrent de façon marquée. Au sein du lac, les quatre types semblent s'être différenciés par rayonnement trophique mais devraient être considérés conspécifiques.

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Life histories of organisms appear to be adapted to the environment through natural selection, similar to what is known for morphological characters (Fisher 1930; Schaffer 1974; Stearns 1976, 1980). For example, experiments with mosquitofish (*Gambusia affinis*) (Stearns 1983, 1984), cutthroat trout (*Salmo clarki*), and Dolly Varden charr (*Salvelinus malma*) (Jonsson et al. 1984) indicate that age and size at sexual maturity change in response to changes in the environment. Schaffer and Elson (1975) gave circumstantial evidence that changes in age at sexual maturity of Atlantic salmon (*Salmo salar*) in North America in recent years were due to environmentally induced changes in genetics.

Within lakes, Arctic charr (*Salvelinus alpinus*) exhibit up to four variants (Behnke 1980, 1984). Different morphs may be distinguished by a number of traits, such as choice of food and habitat, individual growth rate, age and size at sexual maturity, time and place of spawning, body proportions, coloration, and parasites (Balon 1980; Johnson and Burns 1984). Arctic charr, like many other fish species, develop morphs through trophic differentiation (or ecological polymorphism) within breeding populations (Skreslet 1973; Sage and Selander 1975; Campbell 1979; Turner and Grosse 1980; Hindar and Jonsson 1982; Kornfield et al. 1982; Grudzien and Turner 1984). Under such conditions, a single population may split into several genetically different local stocks (Kirkpatrick and Selander 1979; Ryman et al. 1979; Ferguson and Mason 1981; Ryman 1981; Hindar et al. 1986), which at least in salmonids may be based on their well-documented reproductive homing behavior (Stuart 1953, 1957; Frost 1965; Behnke 1972). From the perspective of evolutionary genetics, the discontinuous trophic variation found in Arctic charr may well be the type of niche-specific polymorphism that, for example, Maynard Smith (1966) and Turner and Grosse (1980) have regarded as a fundamental prerequisite for sympatric speciation.

Arctic charr in Thingvallavatn (the name means lake by the Thingvellir, the original site of the 1000-yr-old Icelandic Parliament) exhibit four phenotypical and ecological variants (Sæmundsson 1904; Snorrason 1982; Skúlason 1983; Malmquist et al. 1985, 1986). These morphs are small and large benthivorous charr and planktivorous and piscivorous charr (Table 1). The benthivorous morphs live in the littoral zone. Small benthivorous charr stay in small crevices and under lava stones in the substratum during the day, but at least some of them occur in the epibenthic zone at night. Large benthivorous charr are epibenthic. Both feed mainly on the snail *Lymnaea peregra*. Planktivorous and piscivorous charr occur in both pelagic and epibenthic habitats. Planktivorous charr feed mainly on crustacean zooplankton and chironomid pupae. Piscivorous charr prey mainly on threespine sticklebacks (*Gasterosteus aculeatus*), but may also take small charr (Malmquist et al. 1985; Sandlund et al. 1987).

Observations on the spawning grounds have shown that phenotypically similar fish mate; the morphs overlap only partly in spawning time (Table 1: Skúlason 1983), and no natural crossbreeding between morphs has been observed (K. Gunnarsson and H. Sigurjónsdottir, Institute of Biology, University of Iceland, Grensásvegur 12, IS-108 Reykjavik, Iceland, pers. comm.).

Morphologically, the benthivorous morphs have overshot mouths, and planktivorous and piscivorous morphs have terminal mouths. This inherited difference is significant soon after the onset of external feeding (Skúlason 1986). The number of gill rakers is significantly different among all morphs except between piscivorous and planktivorous charr (Table 1). These morphological differences among morphs indicate that they are genetically different. However, based on electrophoretic analysis of the protein products of 39 loci, the morphs are very closely related. The values of Nei's genetic distance range from 0.00004 to 0.00126 (Magnússon and Ferguson 1987). Although the genetic differentiation between small benthivorous charr and the other three morphs is significant, Magnússon and Ferguson (1987) concluded that they are conspecifics and do not represent different evolutionary lineages. The small genetic differences between the morphs indicate that their separation, if any, is very recent.

Life history variables of the four morphs may be adapted separately, through natural selection, to their respective habitats, assuming that they are reproductively isolated. If so, we predict that small benthivorous charr should be more slow growing than large benthivorous charr. Small benthivorous charr should be able to move freely within the stone matrix of the lava bottom. Large benthivorous charr, living on the bottom, face a higher number of potential predators. Through high growth rates, predation risk is decreased (Nikolsky 1963).

TABLE 1. Morphological and ecological characteristics of the four Arctic charr morphs in Thingvallavatn. Data from Malmquist et al. (1985), Sandlund et al. (1987), and S. S. Snorrason et al. (unpubl. data).

| Morph | Phenotypic characterization | Mean number of gill rakers | Spawning period | Habitat | Main food |
|---|---|---|---|---|---|
| Small benthivorous | Overshot mouth, adult fish length 7 – 31 cm, parr marks along the flanks, dark coloration | 25.2 | July – November | Littoral | *Lymnaea peregra*, chironomid larvae |
| Large benthivorous | Overshot mouth, adult fish length 9 – 55 cm, no parr marks, dark coloration | 25.9 | July – August | Littoral | *Lymnaea peregra* |
| Planktivorous | Terminal mouth, adult fish length 13 – 26 cm, no parr marks, silvery coloration | 27.4 | September – October | Whole lake | Crustacean zooplankton, chironomid pupae |
| Piscivorous | Terminal mouth, adult fish length 23 – 65 cm, no parr marks, light to silvery coloration | 27.1 | September – October | Whole lake | Threespine stickleback (*Gasterosteus aculeatus*) |

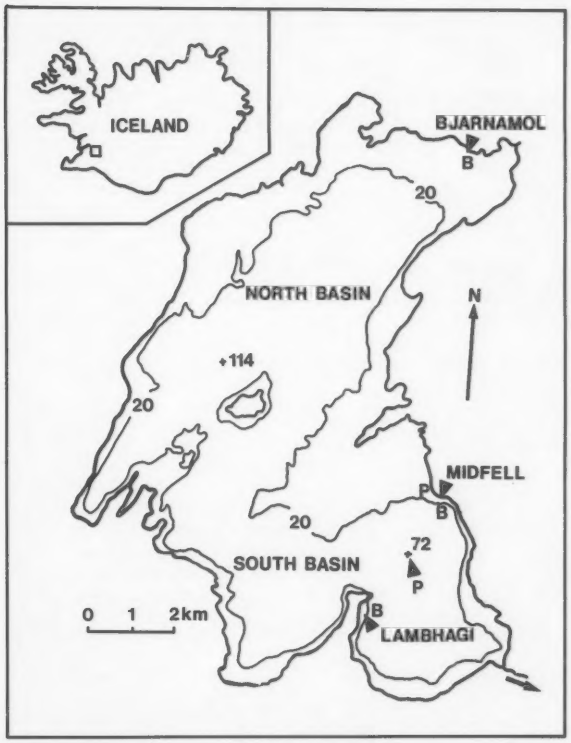


FIG. 1. Thingvallavatn with 20-m depth contour. Locations of benthic (B) and pelagic (P) gillnet fishing are indicated.

Planktivorous and piscivorous charr should be even more fast growing, as the risk of predation is even higher in the limnetic zone than in the structured littoral zone (Crowder and Cooper 1982). The age at maturity of the various morphs should be close to the inflection points of their respective growth curves, so that their net reproductive rates are maximized (Stearns 1976, 1980; Jonsson et al. 1984; Jensen 1985). Furthermore, we predict that the annual reproductive investment among sexually mature fish is higher for planktivorous and piscivorous charr than for the benthivorous morphs. The latter prediction is based on differential mortality expectations in the various habitats as indicated above. Therefore, the following null hypothesis was tested: life history traits such as growth rate, age at sexual maturity, sex ratios, and size-related annual reproductive investment do not differ among morphs.

## Methods

### Study Area

Thingvallavatn (64°6′N, 21°8′W, 101 m a.s.l.) is the largest lake in Iceland (Fig. 1), with an area of 84 km², mean depth of 34.1 m, and maximum depth of 114 m (Rist 1975). The lake is situated in a fault valley in an area of tectonic and geothermal activity. Apart from a large lava flow from the northeast, approximately 9000 yr ago, sporadic tectonic activity and minor local volcanic eruptions have disturbed parts of the lake basin, especially the northern basin. The major disturbances have occurred at intervals of several thousand years (Sæmundsson 1965). The southern basin has most probably remained unaltered since the glacial retreat about 11 500 yr ago (Fig. 1).

Approximately 90% of the inflow to the lake is subterranean through the porous lava rocks in the catchment area (Rist and Ólafsson 1986). The nature of the water inflow along with the lake morphometry and rather windy climate (Einarsson 1988) stabilize both the annual water temperature amplitudes and the chemical composition of the lake (Snorrason 1982). The temperature varies between 0 and 11°C during the year, with a maximum in the uppermost 25 m in July–August. Specific conductivity ($\kappa_{20}$) is approximately 65 $\mu S \cdot cm^{-1}$ (Ólafsson 1980), and Secchi disc transparency is 6–15 m in summer. The lake was dammed in 1959, and since then the water level has fluctuated less than 50 cm during the year.

Thingvallavatn has a large pelagic zone and a benthic zone consisting of three main strata: (1) a littoral zone consisting of lava and hard bottom often with loose boulders and rocks at 0–10 m depth, (2) a littoral soft bottom covered by dense stands of *Nitella opaca* at 10–20 m depth, and (3) a profundal soft bottom of diatomic gyttja (Lindegaard 1980; Lastein 1983) at depths below 20 m. The zoobenthos is dominated by chironomid larvae, tubificids, and molluscs. Maximum densities of chironomids and molluscs are found in the stony littoral zone (0–10 m) (Lindegaard 1980; Snorrason 1982). The zooplankton are predominantly *Daphnia longispina*, *Cyclops abyssorum medianus*, and *Leptodiaptomus minutus* (Antonsson 1977). The lake supports a dense population of Arctic charr as well as threespine sticklebacks and a few brown trout (*Salmo trutta*). Local farmers annually fish between 7 and 70 metric tons of planktivorous charr on the spawning grounds, which amounts to approximately 30% of the biomass of adult planktivorous charr (T. Lindem et al., unpubl. data). Large benthivorous and piscivorous charr are also fished to some degree (Kristjánsson and Adalsteinsson 1984). Among small benthivorous charr, only the very oldest and largest are fished as a by-product of the planktivorous charr fisheries. The ecosystem of Thingvallavatn thus appears to be very stable for the subarctic region (Snorrason 1982; Jónasson and Lindegaard 1988).

### Data Collection and Analysis

Arctic charr were collected ($N$ = 6332) from Thingvallavatn during three periods: August 1983, May–June 1984, and August–December 1984. Fish were caught with benthic and pelagic nets. The benthic nets were 1.8 m and the pelagic nets 6 m in vertical dimension. The nets were gangs of panels with 12 different bar mesh sizes: 10, 12.5, 15, 18.5, 22, 25, 29, 33, 38, 43, 50, and 60 mm. Benthic nets were used in three locations (Fig. 1) during at least two 24-h periods in each sampling period on the following substrata and depth zones: (1) stony bottom, 0–10 m; (2) *Nitella* bottom, 10–20 m; and (3) soft bottom, 20–35 m. Pelagic nets were set in two locations (Fig. 1) at 0–6, 6–12, 20–26, 35–41, 50–56, and 66–72 m for at least two 24-h periods at each depth zone and sampling series. In the *Nitella* zone at Midfell, pelagic nets were set for one 24-h period in each sampling series in depth zones 0–6 and on the bottom at 7–13 m (Fig. 1). Pelagic nets were lowered along ropes anchored on the bottom and kept at the correct depths by a combined use of weights and floats. More details about sampling are given in Sandlund et al. (1987). The nets used caught fish from 7 to 48 cm in length.

Arctic charr were classified into morphs on the basis of body length, upper and lower jaw lengths, head shape, and color

TABLE 2. Ovarian weight (OW, g) on individual fecundity ($F$) of Arctic charr morphs from Thingvallavatn. All coefficients of determination ($R^2$) are significant ($P < 0.05$). Data from Skúlason (1983).

| Morph | Regression | Fecundity range | $R^2$ | $N$ |
|---|---|---|---|---|
| Small benthivorous | $\log_e OW = 0.01045F + 0.0665$ | 13–385 | 0.70 | 46 |
| Large benthivorous | $\log_e OW = 0.00087F + 2.9640$ | 135–2974 | 0.77 | 21 |
| Planktivorous | $\log_e OW = 0.00373F + 1.7468$ | 90–297 | 0.55 | 26 |
| Piscivorous | $\log_e OW = 0.00094F + 3.0872$ | 293–2076 | 0.89 | 12 |

TABLE 3. Fork length ($L$, mm) on otolith radius ($r$, mm) of morphs of Arctic charr from Thingvallavatn. All coefficients of determination ($R^2$) are highly significant ($P < 0.001$)

| Morph | Regression | Length | $R^2$ | $N$ |
|---|---|---|---|---|
| Small benthivorous | $\log_e L = 7.27 - 2.29 \log_e r + 0.37(\log_e r)^2$ | 73–310 | 0.76 | 232 |
| Large benthivorous | $\log_e L = 4.60 - 1.25 \log_e r + 0.28(\log_e r)^2$ | 70–470 | 0.93 | 681 |
| Planktivorous | $\log_e L = -33.28 + 15.24 \log_e r - 1.51(\log_e r)^2$ | 85–260 | 0.76 | 4467 |
| Piscivorous[a] | $\log_e L = -6.86 + 3.75 \log_e r - 0.26(\log_e r)^2$ | 85–480 | 0.80 | 3406 |

[a]Planktivorous charr were used for fish younger than 6 yr.

patterns (see unpublished data of S. S. Snorrason et al. for details). Furthermore, fork length ($L$, millimetres), weight ($W$, grams), sex, and degree of sexual maturity (Dahl 1917) were recorded for each fish (Table 1). Morph characterization of small fish was sometimes uncertain, as small benthivorous charr may resemble small individuals of the large benthivorous morph. Furthermore, small piscivorous charr are difficult to distinguish from planktivorous charr. Ages were determined from otoliths (Nordeng 1961; Christensen 1964). Individual fecundities were recorded as number of eggs of females in maturity stages 4–5. Age-specific morph fecundities ($PF_i$, percent) were estimated as

$$(1)\quad PF_i = N_i F_i \, 100 / \sum_{x=a}^{n} N_x F_x$$

where $N_i$ is number of mature females in the $i$th age group of a morph, $F_i$ is the corresponding age-specific fecundity estimated from mean lengths of the fish, $a$ is the age of the youngest sexually mature female, and $n$ is the age of the oldest mature female. Gonadosomatic indexes (GSI) of females were estimated as ovarian weight on somatic weight (SW, grams):

$$(2)\quad GSI = OW\ 100/SW$$

where SW = W − OW. Regressions of ovary weight (OW, grams) on fecundity are based on data of Skúlason (1983) (Table 2). Relative annual investment in reproduction ($RAI_i$) of females at age $i$ was estimated as (Mills and Eloranta 1985)

$$(3)\quad RAI_i = OW_i/OW_i + ISW_i$$

where ISW is yearly increase in somatic weight. The estimation is based on mean wet weights and percentages of mature and immature females in the various age groups. Annual survival rates were estimated using the Chapman–Robson method (Youngs and Robson 1978). Empirical growth rates were calculated using von Bertalanffy's function (Allen 1966)

$$(4)\quad L_A = L_\infty(1 - e^{-kA})$$

where $L_A$ is fork length at age $A$, $L_\infty$ is asymptotic length, and $k$ is a constant. von Bertalanffy's equation is not used in its more general form where the exponent $kA$ is replaced by $k(A - A_0)$ because very few charr smaller than 7 cm were sampled. However, $A_0$ is generally small and has little effect (Roff 1984). Back-calculation of growth was performed using otoliths measuring $r_1$, defined by Jonsson and Stenseth (1977), as the independent variable (Table 3). Weight–length relationships were estimated from wet weights ($W$, grams) and fork lengths ($L$, millimetres) in the regression model

$$(5)\quad \log_e W = a \log_e L + b$$

where $a$ and $b$ are constants.

Tests for significant differences between sexes in age at sexual maturity were based on the normal approximation to the binomial distribution (Siegel 1956), with percentage by numbers as the data input. A two-sample test is expressed by

$$(6)\quad z^* = \sum_{i=a}^{b} t^2$$

where

$$t_i = \sqrt{n_i m_i/(n_i + m_i)} \cdot (p_i - q_i)/\sqrt{r_i\,(1 - r_i)}.$$

$$p_i = x_i/n_i$$

$$q_i = y_i/m_i$$

TABLE 4. Adult annual rate of survival ($S$) ± 95% confidence limits, $\chi^2$ test for goodness of fit to the Chapman–Robson model, and sample sizes of the various Arctic charr morphs from Thingvallavatn based on catch data from August–September 1983 and 1984. $\chi^2$ values are not significant ($P > 0.05$), indicating agreement between sample observations and the model used.

| Morph | Year | $S$ | $\chi^2$ | Age (yr) | Sample size |
|---|---|---|---|---|---|
| Small benthivorous | 1983 | 0.63 ± 0.04 | 0.078 | 5–13 | 209 |
| | 1984 | 0.51 ± 0.04 | 2.887 | 6–11 | 164 |
| | Pooled | 0.57 ± 0.03 | 3.255 | 5–13 | 488 |
| Large benthivorous | 1983 | 0.65 ± 0.05 | 0.037 | 6–14 | 112 |
| | 1984 | 0.59 ± 0.07 | 0.004 | 6–14 | 72 |
| | Pooled | 0.63 ± 0.05 | 0.911 | 7–15 | 145 |
| Planktivorous | 1983 | 0.47 ± 0.03 | 1.890 | 6–14 | 501 |
| | 1984 | 0.41 ± 0.01 | 0.074 | 5–13 | 1789 |
| | Pooled | 0.43 ± 0.02 | 0.572 | 6–14 | 2179 |
| Piscivorous | 1983 | 0.63 ± 0.06 | 0.063 | 7–15 | 89 |
| | 1984 | 0.62 ± 0.07 | 0.221 | 7–14 | 78 |
| | Pooled | 0.63 ± 0.05 | 0.030 | 7–15 | 169 |

$$r_i = (x_i + y_i)/(n_i + m_i)$$

where $x_i$ and $y_i$ are the numbers of mature males and females at age $i$, $n_i$ and $m_i$ are the numbers of all males and females at age $i$, $t_i$ is $t$ distributed with $(n_i + m_i - 2)$ degrees of freedom, and $z^*$ is $\chi^2$ distributed with $(b + 1 - a)$ degrees of freedom.

## Results

### Survival, Growth, and Size

Annual survival rate of adults was lowest for planktivorous charr (0.41–0.47) (Table 4). For small benthivorous, large benthivorous, and piscivorous charr, it was 0.51–0.65. Adult age groups which yielded a significant ($P < 0.05$) fit to the Chapman–Robson model were used for the analysis. The data indicated that the survival rate was higher in 1983 than in 1984 among small benthivorous charr, large benthivorous charr, and planktivorous charr ($t$-tests, $P < 0.05$).

The mean size of mature individuals increased from small benthivorous charr through planktivorous and large benthivorous charr to piscivorous charr (Table 5). The range in length of adult large benthivorous charr was larger than that of the other adult morphs.

The small benthivorous morph was the slowest growing, and growth rates were successively higher in large benthivorous, planktivorous, and piscivorous charr. The growth rates levelled off at approximately 13, 20, and 30 cm in small benthivorous, planktivorous, and piscivorous charr, respectively. Piscivorous charr, feeding on planktivorous charr (cannibals), were sometimes caught in the lake, and such individuals may become larger than 60 cm in length (Malmquist et al. 1985). In the present samples, however, there were no piscivorous charr larger than 48 cm (Table 5). Large benthivorous charr continued to increase in length ($L_{LB}$, millimetres) with increasing age ($A$, years) at an almost rectilinear rate:

$$(7)\quad L_{LB} = 26.3A + 51.3 \qquad N = 644,\ R^2 = 0.80,\ P < 0.0001.$$

Asymptotic length ($L_\infty$) and Brody's growth coefficient ($k$) of von Bertalanffy's growth model (Equation (4)) differed significantly between morphs (Table 5). Fish that were classified as intermediate between small benthivorous and large benthivorous charr had $L_\infty \pm$ SD = 200 + 47 mm ($N$ = 48). Those that on the basis of external morphology were classified as intermediate between planktivorous and piscivorous charr had $L_\infty$ = 229 ± 9 mm ($N$ = 15).

Back-calculation of growth using otoliths (Fig. 2) corresponded well with empirical growth rates (Table 5). The growth curves of planktivorous and piscivorous charr were almost parallel between 1 and 3 yr of age, but planktivorous charr ceased growing in length at a considerably earlier age than piscivorous charr. The back-calculated mean lengths of small benthivorous and large benthivorous charr were significantly different at all ages ($t$-tests, $P < 0.001$). There was no significant difference in growth rate between sexes or sexually immature and mature fish within morphs.

### Weight–Length Relationships

Weight–length relationships show the increase in weight relative to length; the higher the regression coefficient ($a$, Equation (5)), the quicker weight will increase with length. This relationship differed significantly among the morphs in both spring and autumn (test of homogeneity of regression coefficients, June: $F_{3,670} = 64.68$, $P < 0.0001$; August: $F_{3,3760} = 8.685$, $P < 0.05$). Significantly different weight–length relationships were found between the sexes within small benthivorous and planktivorous charr and spring and autumn samples within planktivorous and piscivorous charr (Table 6). Among small benthivorous and planktivorous charr, intramorph regression coefficients decreased with age within age groups 2–9 yr, whereas in large benthivorous and piscivorous charr, it did not (Fig. 3). A significant heterogeneity in regression coefficients among age groups was found only for planktivorous charr ($F_{3,2721} = 7.49$, $P < 0.001$). This means that weight increase with increasing length is smaller among older age groups of small benthivorous and planktivorous charr than in large benthivorous and piscivorous charr.

### Age and Sex

Age at sexual maturity differed between morphs (Fig. 4). Age at maturity of each of the four morphs did not differ between 1983 and 1984 ($P > 0.05$), and they were therefore pooled. Small benthivorous (2–17 yr of age) and planktivorous charr (1–14 yr of age) both matured at an early age and over a few age groups. Among small benthivorous charr, more than 50% of the males in age group 2 were mature, and 50% of the females in age group 4 were mature. The difference between sexes was significant ($z^* = 105.34$, 4 df, $P < 0.0001$). Among planktivorous charr, more than 50% of the males in age group 4 and females in age group 5 were mature. This difference between sexes was also highly significant ($z^* = 508.26$, 5 df, $P < 0.0001$). Large benthivorous (2–18 yr of age) and piscivorous charr (5–18 yr of age) matured later and over a wider range of years. Among large benthivorous charr, 50% maturity of both sexes was attained at age 8 and among piscivorous charr at age 6. Age at maturity differed between sexes for piscivorous charr ($z^* = 24.69$, 5 df, $P < 0.001$), but not for large benthi-

TABLE 5. Empirical sizes (mm) and the parameters (±95% confidence limits) $L_\infty$ (mm) and $k$ of the exponential growth model $L_A = L_\infty(1 - e^{-kA})$ where $A$ is age (yr) of Arctic charr from Thingvallavatn, based on pooled data from August–September in 1983 and 1984.

| | Adult fork length | | | | | | |
|---|---|---|---|---|---|---|---|
| | Males | | Females | | | | |
| Morph | Mean | Range | Mean | Range | $L_\infty$ | $k$ | $N$ |
| Small benthivorous | 96 | 73–260 | 109 | 75–310 | 132.9± 6.7 | 0.333±0.045 | 746 |
| Large benthivorous | 240 | 135–460 | 274 | 90–470 | 553.5±74.2 | 0.086±0.016 | 644 |
| Planktivorous | 175 | 130–230 | 188 | 140–260 | 205.1± 1.6 | 0.417±0.011 | 4224 |
| Piscivorous | 268 | 180–480 | 295 | 200–440 | 302.4±15.1 | 0.328±0.064 | 224 |

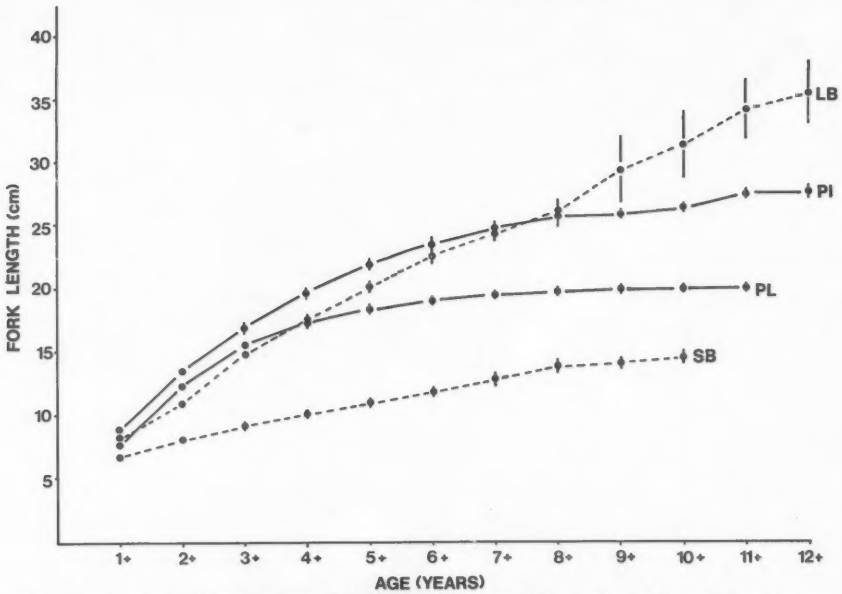


FIG. 2. Back-calculated fork lengths with 95% confidence limits of the estimates for small benthivorous (SB), large benthivorous (LB), planktivorous (PL), and piscivorous (PI) Arctic charr caught in Thingvallavatn, August–September 1983 and 1984.

TABLE 6. Regression constants ($a$ and $b$) and coefficient of determination ($R^2$) of total weight ($W$, g) on fork length ($L$, mm) of Arctic charr morphs from Thingvallavatn caught in 1984. The regression model is $\log_e W = a \log_e L - b$. $N$ = sample size; $F$-values for test of homogeneity between regression coefficients ($a$) between sexes and times of the year: n.s. = $P > 0.05$, *$P < 0.05$, ***$P < 0.001$.

| | | | Regression | | | $F$ | | |
|---|---|---|---|---|---|---|---|---|
| Morph | Month | Sex | $a$ | $b$ | $R^2$ | Sex | Month | $N$ |
| Small benthivorous | August | Male | 3.06 | 11.58 | 0.90 | 5.91* | | 244 |
| | | Female | 2.83 | 10.51 | 0.92 | | | 217 |
| | | Pooled | 2.94 | 11.03 | 0.91 | | | 462 |
| | June | Pooled | 3.06 | 11.78 | 0.95 | | 1.08 n.s. | 50 |
| Large benthivorous | August | Male | 3.10 | 11.82 | 1.00 | 0.04 n.s. | | 162 |
| | | Female | 3.10 | 11.84 | 0.99 | | | 133 |
| | | Pooled | 3.11 | 11.87 | 1.00 | | | 313 |
| | June | Pooled | 3.14 | 12.16 | 0.99 | | 1.40 n.s. | 102 |
| Planktivorous | August | Male | 3.04 | 11.59 | 0.96 | 15.86*** | | 1733 |
| | | Female | 3.11 | 11.97 | 0.97 | | | 1049 |
| | | Pooled | 3.08 | 11.82 | 0.97 | | | 2801 |
| | June | Pooled | 2.40 | 8.43 | 0.81 | 1552.69*** | | 479 |
| Piscivorous | August | Male | 3.20 | 12.44 | 0.97 | 1.27 n.s. | | 67 |
| | | Female | 3.07 | 11.78 | 0.93 | | | 35 |
| | | Pooled | 3.11 | 11.95 | 0.95 | | | 102 |
| | June | Pooled | 3.22 | 12.66 | 0.94 | | 4.87* | 47 |

vorous charr ($z^* = 8.52$, 4 df, $0.1 > P > 0.05$). Some non-breeding fish, males in particular, may have been previous spawners which did not mature in some intervening years.

Sex ratios were investigated during spring (May–June). August–September samples were omitted because the various morphs spawn between July and November (Table 1), and during spawning, sex ratios in gillnet catches may be influenced by differences in activity between sexes (Alm 1959; Jonsson and Hindar 1982). Females predominated among small benthivorous charr (male to female (M/F) = 0.36, binomial test, $N = 49$, $Z = 3.14$, $P < 0.01$) at all ages. Among large benthivorous charr, males were most frequent in most age groups. The M/F ratio of the total sample of large benthivorous charr, however, was not significantly different from unity (M/F =

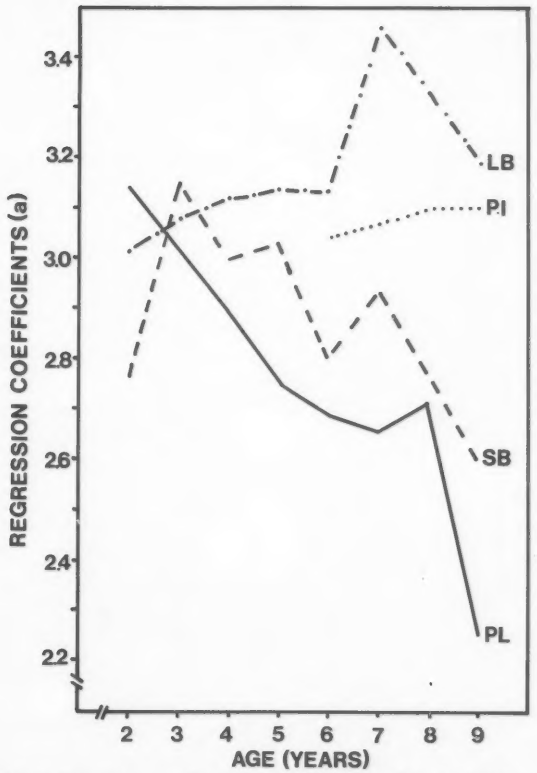


FIG. 3. Regression coefficients ($a$) of the weight ($W1$, g) – fork length ($L$, mm) relationship, $\log_e W = a \log_e L + b$, for each age group within small benthivorous (SB), large benthivorous (LB), planktivorous (PL), and piscivorous (PI) Arctic charr caught in Thingvallavatn, August–September 1983 and 1984. The higher the value of $a$, the greater increase in fish weight with increasing length.

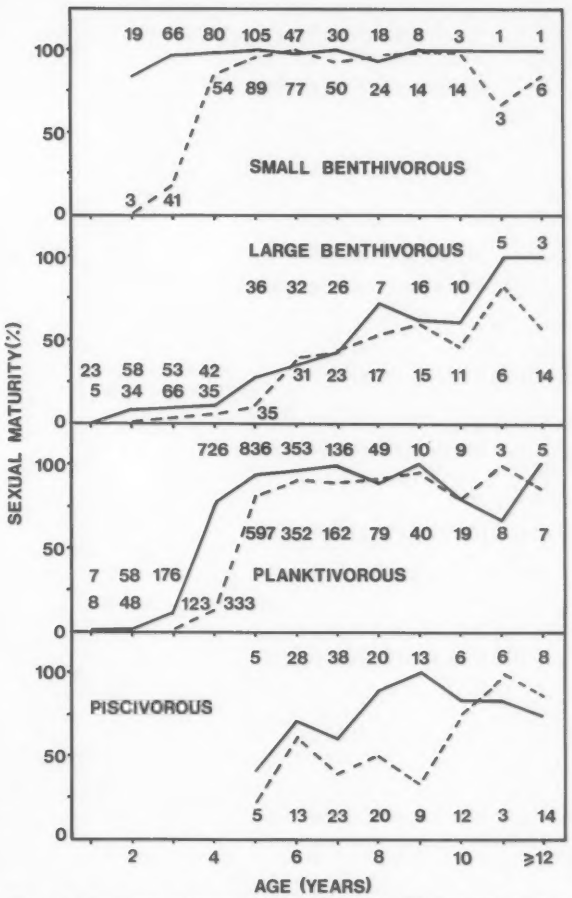


FIG. 4. Percentage of sexually mature males (solid line) and females (broken line) of the four morphs of Arctic charr caught in Thingvallavatn, August–September 1983 and 1984. Female sample size is given below the corresponding male value.

1.38, $N = 100$, $P < 0.15$). Among planktivorous charr the sex distribution approached equality for age groups 2–4 (M/F = 1.29, $N = 176$, $P < 0.15$). In older fish, females were most abundant (M/F = 0.54, $N = 291$, $P < 0.001$). Among piscivorous charr, females predominated in most age groups, but the overall M/F ratio was not significantly different from unity (M/F = 0.57, $N = 47$, $0.1 > P > 0.05$).

## Fecundity and Reproductive Effort

Individual fecundity of females ($F$) increased significantly with fork length ($L$, millimetres) for all morphs (Table 7). Regression analyses were performed with both untransformed and log-transformed data sets, and the data set giving the better fit was used. In the case of planktivorous charr, the predictive value of the individual regressions using length as the independent variable was low. Adding age as a second independent variable increased the coefficent of determination, but it was still low ($R^2 = 0.22$) due to high individual variability and small size range. Length-specific fecundity of small benthivorous charr also decreased significantly with age ($P < 0.0001$), but there were insufficient data to test this relationship for large benthivorous and piscivorous morphs ($P > 0.05$).

The age-specific morph fecundity gives the relative contribution to the total female fecundity of the different age groups within each morph (Fig. 5). In planktivorous charr, contributions to the population fecundity were mostly based on age groups 5–7 whereas in the other morphs, more age groups were involved. Some of the variation in relative contribution between age groups of large benthivorous and piscivorous charr was probably due to small sample sizes.

Gonadosomatic indexes (GSI, Equation (2)) of females decreased with age for all morphs, but were generally higher for planktivorous and piscivorous charr than for small benthivorous and large benthivorous charr (Fig. 6).

Relative annual investment (RAI, Equation (3)) in gonad and somatic growth by females was estimated from mean gonad weight and annual increase in somatic tissues. RAI was less than 0.5 for large benthivorous charr, and for small benthivorous charr younger than 7 yr, and it was 0.7 and higher for planktivorous, piscivorous, and small benthivorous charr 7 yr and older (Fig. 7).

TABLE 7. Individual fecundity on length ($L$, mm) of large benthivorous and piscivorous Arctic charr and on length and age ($A$, yr) of small benthivorous and planktivorous Arctic charr from Thingvallavatn. All regressions are significant ($F$-tests, $P < 0.0001$). $N$ = sample size, $R^2$ = coefficient of determination.

| Morph | Regression | Fecundity range | $R^2$ | $N$ |
|---|---|---|---|---|
| Large benthivorous | $\log_e F = 2.719 \log_e L - 8.97$ | 149–1914 | 0.95 | 7 |
| Piscivorous | $\log_e F = 2.841 \log_e L - 9.70$ | 278–1795 | 0.68 | 26 |
| Small benthivorous | $F = 1.28L - 74.74$ | 12–221 | 0.69 | 175 |
| | $F = 1.38L - 4.75A - 57.91$ | 12–221 | 0.71 | 172 |
| Planktivorous | $F = 1.47L - 57.53$ | 96–431 | 0.11 | 404 |
| | $F = 2.08L - 18.6A - 66.11$ | 96–431 | 0.22 | 397 |
| Pooled | $\log_e F = 2.284 \log_e L - 6.63$ | 12–1914 | 0.89 | 612 |

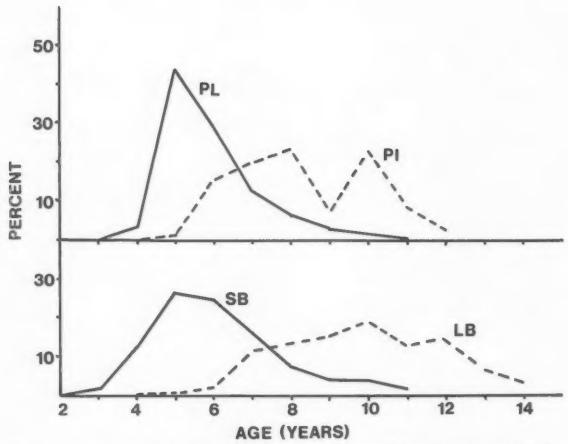


FIG. 5. Age-specific morph fecundities of small benthivorous (SB), large benthivorous (LB), planktivorous (PL), and piscivorous (PI) Arctic charr caught in Thingvallavatn, August–September 1983 and 1984.

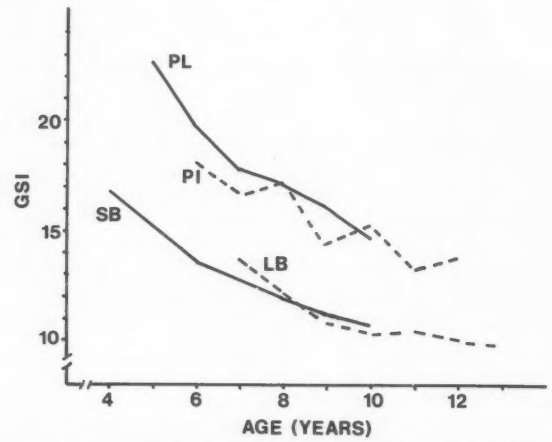


FIG. 6. Gonadosomatic index (GSI) within age groups of small benthivorous (SB), large benthivorous (LB), planktivorous (PL), and piscivorous (PI) Arctic charr caught in Thingvallavatn, August–September 1983 and 1984.

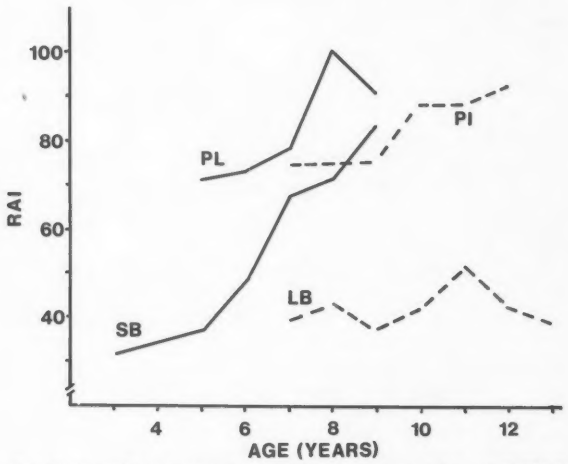


FIG. 7. Annual investments in gonadal relative to somatic growth (RAI) of age-groups of small benthivorous (SB), large benthivorous (LB), planktivorous (PL), and piscivorous (PI) Arctic charr caught in Thingvallavatn, August–September 1983 and 1984.

## Discussion

### Life History Variation of the Morphs

Significant differences among morphs were found in several life history traits including individual growth rate, asymptotic length, age at maturity, GSI, and RAI. Thus, we reject our null hypothesis that the morphs are equal and conclude that the Arctic charr population in Thingvallavatn consists of four different morphs.

Based on the assumption that the morphs through natural selection are adapted to different habitats, we predicted that small benthivorous charr would be more slow growing than large benthivorous charr and that piscivorous and planktivorous charr should be more fast growing at early age than the benthivorous morphs. We also predicted that the age at sexual maturity should be close to the inflection points of the growth curves, i.e. planktivorous and small benthivorous charr should mature early, and piscivorous and large benthivorous charr should mature at old age. Moreover, RAI was expected to be higher in piscivorous and planktivorous charr occupying pelagic waters than in the benthivorous morphs living in the more

sheltered littoral zone. This prediction was based on differences in expected mortalities of fish living in structured and unstructured habitats (Charnov et al. 1977; Crowder and Cooper 1982). Our results are in accordance with these expectations. However, we were not able to measure juvenile mortality. Adult mortality rate was higher for the planktivorous morph than for the other morphs. One of the reasons for this may be the high fishing rate in the lake.

Differences in age at maturity cause some of the variation in adult sizes among the morphs, as maturity influences the individual growth rate of the fish. The growth rate decreases when maturity is attained because the energy expenditure to gonadal development and reproductive activity reduces somatic growth (Calow 1979; Reznick and Endler 1982; Roff 1983). Thus, early-maturing fish become small and late-maturing ones may grow large. Therefore, differences in age at sexual maturity also partly explain the observed differences in adult body size between planktivorous, piscivorous, and small and large benthivorous charr.

Differences in adult sizes of planktivorous and piscivorous charr are probably also due to differences in egg size and diet. Piscivorous charr have larger eggs than planktivorous charr (Skúlason 1983), and their offspring have consequently a growth and size advantage during the first weeks (Kazakov 1981; Kamler and Kato 1983; Skúlason 1986). Growth rates of these two morphs are parallel during the 3 first yr of life, and piscivorous charr develop probably from the fastest growing individuals that when young are classified as planktivorous charr. Piscivorous charr switch usually from plankton to fish feeding at a fork length of approximately 23 cm (Malmquist 1988), and planktivorous fish that reach this threshold length may become piscivorous. Most planktivorous charr, however, never grow that large and remain plankton feeders.

Size differences between small and large benthivorous charr may also partly originate from differences in egg size, as the smaller morph has smaller eggs than the large one. Diet differences, on the other hand, should not account for differences in adult size, as both morphs largely feed on *Lymnaea peregra*. However, they do occupy different feeding habitats (Sandlund et al. 1987). Small benthivorous charr live sheltered within the stone matrix of the lava bottom whereas the large benthivorous charr live more exposed upon the substratum. A small body size may be advantageous for fish living within the substratum because the interstitial spaces are limited and small individuals are able to maneuver more freely among the stones than large fish. We do not know any charr predator feeding on charr within the bottom substratum. For charr living upon the bottom substratum, a large body size may be advantageous because foraging efficiency is probably positively correlated with size, and the risk of predation decreases with size (Nikolsky 1963). Medium-sized fish may do relatively poorly in both habitats. Under such conditions, fish experiencing different growth rates and body sizes may select different habitats, and natural selection may favor early age at maturity among slow-growers and old age among fast-growers. In this way, adaptation to life in two different habitats may influence the growth and size differences between the two benthivorous morphs in Thingvallavatn. Thus, diet, habitat choice, and egg size may be important for morph differentiation of the Arctic charr in Thingvallavatn.

Individual fecundity varied among morphs; it is lowest in small benthivorous charr and highest among large benthivorous charr and piscivorous charr. The main reason is that fecundity increases with fish size. The benthivorous fish invested less in gonadal than somatic growth compared with the planktivorous and piscivorous morphs. Furthermore, the length-specific fecundity decreased with age in planktivorous and small benthivorous charr, even though the relative amount of energy allocated to gonadal development increased with age. Reduction in length-specific fecundity with increasing age has also been found in other populations of Arctic charr (Jonsson and Hindar 1982) and in other salmonids as brown trout (Jonsson 1985).

The relative amount of energy allocated to gonadal production increased with age in all morphs. Increased investment in reproductive relative to somatic growth with age may be a general trend among adult iteroparous fish species until they reach senility (Nikolsky 1963). One reason may be that the chance of surviving another year decreases with age. On the other hand, young fish may show restraint in gonadal production, thus, postspawning mortality decreases and future growth and fecundity increase.

Sex ratios of piscivorous, adult planktivorous, and small benthivorous charr showed a female excess whereas males were more abundant in most age groups of large benthivorous charr. Female excess in the three former morphs may result from earlier age at maturity of males than of females. Natural mortality of fish that are sexually mature is higher than that of immature individuals at the same age (Svärdson 1951; Calow 1979; Jonsson et al. 1984). Among large benthivorous charr the sexes attained maturity at the same age.

The variation in the adult sizes and ages at maturity of the charr morphs in Thingvallavatn embraces a large part of the total variation observed in lake resident stocks of Arctic charr throughout the Holarctic distribution area of the species (e.g. Schmidt-Nielsen 1939; Nilsson 1955; Frost 1965; Nilsson and Filipsson 1971; Adalsteinsson 1979; Jonsson and Østli 1979; Johnson 1980; Jonsson and Hindar 1982; Nordeng 1983; Gydemo 1984; Klemetsen et al. 1985; Hindar et al. 1986). Small benthivorous charr, on one hand, are of the same size and age as the smallest and youngest adult charr observed elsewhere (Johnson 1980). The large size and high age at maturity of piscivorous and benthivorous charr are not uncommon for subarctic populations (Klemetsen and Grotnes 1980; Nilsen and Klemetsen 1984), and even higher ages at maturity have been reported from populations in northern Canada (Johnson 1980) and Bear Island (Klemetsen et al. 1985). No single lake, however, is known to support such a diverse variety of Arctic charr as found in Thingvallavatn.

## Evolution of the Morphs

The charr morphs in Thingvallavatn may have been developed through trophic differentiation within the lake. The idea of trophic radiation in Arctic charr populations within lakes has been advocated by Hindar and Jonsson (1982) and Hindar et al. (1986). In a survey of Arctic charr in Norway, Hindar et al. (1986) documented greater genetic distances between stocks of the same morph in different lakes than between morphs in the same lake, supporting the hypothesis that the morphs have evolved with the lakes. Arctic charr is commonly observed to differentiate into two or three sympatric trophic variants (Johnson 1980). Four sympatric morphs, as in Thingvallavatn, are rare (Behnke 1984). We consider the possibilities of trophic radiation among Arctic charr in this lake as particularly good for the subarctic region for two reasons: (1) the lake offers several different food niches which are relatively stable both within

and between years (see Study Area) and (2) there are only two other freshwater resident fish species in Iceland, brown trout and threespine stickleback. Brown trout is rare in Thingvallavatn due to very restricted spawning grounds. Threespine stickleback is numerous, but mainly restricted to the macrophyte vegetation (*Nitella opaca*) in the 10–20 m depth zone (O. T. Sandlund et al. unpubl. data).

The small genetic differences between the charr morphs in Thingvallavatn (Magnússon and Ferguson 1987) make the multiple invasion theory (Svärdson 1961; Nyman et al. 1981) improbable. This hypothesis claims that sympatric charr morphs are sibling species which evolved allopatrically during the Pleistocene glaciations and in postglacial time have immigrated into some lakes where they now reside as different species.

We do not know to what extent natural cross-breeding occurs among the morphs. However, the morphs overlap only partly in spawning times and areas, and observations of spawning charr indicate that phenotypically similar fish tend to mate with each other (Jonsson and Hindar 1982; K. Gunnarson and H. Sigurjónsdóttir, pers. comm.). Thus the genetic exchange between the morphs may be limited. The genetic difference between the morphs, however, is very small; the value of Nei's genetic distance is only 1/10 of that of Arctic charr populations in eastern and western Norway, which has been geographically isolated at least since the postglacial immigration 10 000 yr ago (Hindar et al. 1986). Thus, if the morphs in Thingvallavatn are reproductively isolated, this separation probably occurred recently.

The present investigation shows that one lake may support several morphs of Arctic charr. The morphic differentiation seems to have occurred through trophic radiation within the lake. Reproductive isolation between the trophic variants may be maintained through behavioral mechanisms. We regard the charr morphs as conspecifics, equivalent to local, sympatric stocks in other salmonids such as brown trout (Ryman et al. 1979; Ryman and Ståhl 1981; Ferguson and Mason 1981).

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# Multivariate Morphometrics in Aquaculture: A Case Study of Six Stocks of the Common Carp (*Cyprinus carpio*) from Italy

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Corti, M., R. S. Thorpe, L. Sola, V. Sbordoni, and S. Cataudella. 1988. Multivariate morphometrics in aquaculture: a case study of six stocks of the common carp (*Cyprinus carpio*) from Italy. Can. J. Fish. Aquat. Sci. 45: 1548–1554.

Multivariate morphometry was used to investigate the distinctness and interrelationships of six stocks of the common carp (*Cyprinus carpio*). A "size" component was clearly identified by multiple group principal component analysis. Canonical variate analysis computed only on the "shape" components showed that the stocks were morphometrically distinct and that the phenetic relationships based on allozymic and morphometric data are highly congruent. We therefore suggest that multivariate morphometrics could represent an appropriate and convenient tool to detect variation between strains in carp culture.

Des techniques morphométriques à plusieurs variables ont été utilisées afin d'étudier le caractère nettement différent de six stocks de carpes communes (*Cyprinus carpio*) et leurs interrelations. Une composante « taille » a été clairement identifiée par l'analyse en composante principale à plusieurs groupes. L'analyse canonique des variables calculées seulement sur les composantes « forme » a montré que les stocks étaient différents du point de vue morphométrique et que les rapports phénétiques fondés sur des données allozymiques et morphométriques sont très pertinents. D'après nous, les mesures morphométriques à plusieurs variables représenteraient un outil utile et approprié permettant de déceler des variations entre les souches de carpe d'élevage.

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The common carp (*Cyprinus carpio*) is a temperate-water fish which is widespread through out the Palearctic region. Historically it has been the primary subject of freshwater fish farming in Europe. It is widely reared and several programmes directed towards artificial selection, and the establishment of gene banks have been planned in the last decade (see Jhingran and Pullin 1985).

In Italy, carp from central and eastern Europe have been, and are, continuously transplanted for restocking and angling purposes so that the original populations (Bonaparte 1832–41) are no longer recognizable, and a generally confused picture of the origin of farmed common carps has emerged. In a previous paper (Cataudella et al. 1987), a multidisciplinary approach (i.e. cytogenetic, allozymic, and morphometric) was used to characterize six groups of common carp from different Italian water bodies in order to determine methods useful for stock characterization, interspecific breeding plans, and selection programmes. Multivariate morphometry contributed to stock characterization, but only limited congruences between genetic (allozymic) and morphometric data sets were found.

The present paper attempts to address the following problems: (1) Since the pattern of morphometric relationships can be perturbed by bias in the growth determined size of the individuals from different stocks, is it possible to detect and remove a "size" component from the data? (2) How congruent is the general pattern of morphometric relationships among the stocks with their allozymic affinities? (3) Is it possible to recognize a stock morphometrically with sufficient confidence to make the technique suitable for use in fish farming research programmes?

Multivariate morphometry has been chosen because it represents an appropriate tool to assess distinctness and phenetic relationships between closely related taxa as in the study of

TABLE 1. Stocks, localities, geographic origin, squamation, number of individuals examined, and average total length with standard deviation. Each locality name is followed by its provincial district. The question mark in CCA reflects its uncertain origin. Squamation is a monomorphic trait in all stocks except CCC and CCF.

| Stock | Locality | Origin | Squamation | Number of specimens | Total length (cm) |
|---|---|---|---|---|---|
| CCA | Fish farm (Grosseto) | ? | Complete | 18 | 22.5 ± 1.3 |
| CCB | Alfieri lake (Latina) | Eastern Europe | Mirror | 13 | 15.5 ± 2.7 |
| CCC | Valdagri Fish Farm (Matera) | Tiber, China | Complete/mirror | 27 | 28.4 ± 5.7 |
| CCD | Land Reclamation Pond (River Reno, Bologna) | Po Plain | Complete | 13 | 43.9 ± 3.9 |
| CCE | Landi Fish Farm (Bologna) | Germany | Leather | 5 | 41.9 ± 20.7 |
| CCF | Power Plant (Milano) | Eastern Europe | Complete/mirror | 16 | 37.5 ± 15.1 |

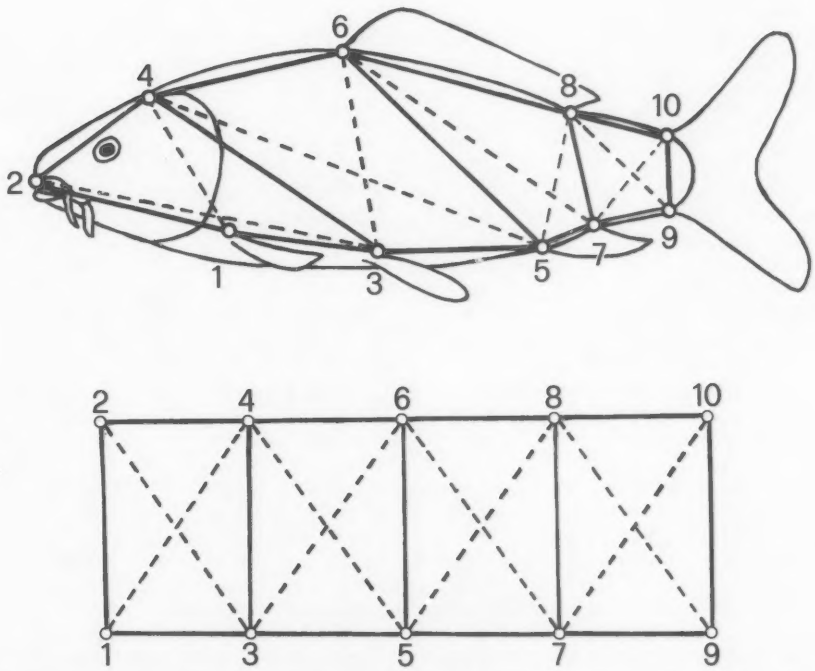


FIG. 1. Data recording procedure. Top: 21 distance characters recorded on X-ray pictures between the following homologous landmarks: 1, upper insertion of pectoral fin; 2, upper end of maxilla; 3, upper insertion of ventral fin; 4, top end of sovraoccipital; 5, insertion first ray of anal fin; 6, insertion first ray of dorsal fin; 7, insertion last ray of anal fin; 8, insertion last ray of dorsal fin; 9, lower insertion of caudal fin; 10, upper insertion of caudal fin. Bottom: Geometric representation of the data recording procedure. Each single cell is determined by four sides and two diagonals. Modified from Strauss and Bookstein (1982).

geographic variation and racial affinities (Gould and Johnston 1972; Reyment et al. 1981; Thorpe 1976, 1983a, 1987). Multivariate morphometry has become a widely used approach in ichthyology to detect patterns of phenetic relationships between taxa (Humphries et al. 1981; Johnson et al. 1983; Libosvarsky and Kux 1982; Shaklee and Tamaru 1981; Strauss and Bookstein 1982; Winans 1984), to assess possible and effective hybridization events (Humphries 1984; Neff and Smith 1978), and to describe development in wild and reared larvae (McGurk 1985).

Since we have to contend with relatively slight morphometric differences between similar stocks of the same species, some

TABLE 2. Multiple group principal component analysis giving the 16 eigenvalues extracted from the pooled within-group covariance matrix and the character coefficients of the first normalized eigenvector which expresses 91.398% of the within-group variance. This vector is associated with the largest eigenvalue (No. 1) and all the coefficients are of the same sign and magnitude, so that this vector was considered as representing "size." Coefficients of the other 15 eigenvectors (not represented) all differ in their sign and magnitude and were considered as representing "shape." Only 16 characters were used out of the original 21, as 5 characters were not informative.

| Eigenvalue | | Coefficient of first normalized eigenvector | Characters between landmarks |
|---|---|---|---|
| 1 | 0.905 | −0.235 | 1–2 |
| 2 | 0.027 | −0.252 | 1–3 |
| 3 | 0.014 | −0.221 | 1–4 |
| 4 | 0.010 | −0.223 | 2–4 |
| 5 | 0.008 | −0.225 | 3–6 |
| 6 | 0.005 | −0.251 | 4–5 |
| 7 | 0.004 | −0.246 | 4–6 |
| 8 | 0.004 | −0.261 | 5–6 |
| 9 | 0.003 | −0.279 | 5–7 |
| 10 | 0.003 | −0.256 | 5–8 |
| 11 | 0.002 | −0.261 | 7–8 |
| 12 | 0.002 | −0.272 | 7–9 |
| 13 | 0.001 | −0.267 | 7–10 |
| 14 | 0.001 | −0.264 | 8–9 |
| 15 | 0.001 | −0.232 | 8–10 |
| 16 | 0.001 | −0.244 | 9–10 |

care has to be taken to identify size and shape components of variation and to record an appropriate set of characters. Size is a component which can, in continuously growing organisms such as bony fish, perturb an assessment of general racial differentiation (Thorpe 1983a, 1983b). Several approaches have been used in attempts to overcome the problem. Libosvarsky and Kux (1982) selected individuals of the same size to analyze morphometric variation between three *Gobio* species. Ratios, where the denominator is standard length, have been used by Johnson et al. (1983) and Shaklee and Tamaru (1981). However, the use of ratios to adjust for size variation has been questioned for many statistical reasons (Atchley et al. 1976; Humphries et al. 1981; Thorpe 1983a). Principal component analysis has been widely used to detect size vectors, usually identified with the first principal component (see Neff and Smith 1978 and Neff and Marcus 1980 for a discussion). Principal component analysis does not require a priori recognition of groups, and if there are several groups, data are pooled irrespective of groups. Humphries et al. (1981) suggested a different multivariate procedure to analyse size and shape, subsequently adopted by Chernoff et al. (1982) and Humphries (1984). Another multivariate technique, multiple group principal component analysis (MGPCA), which can detect a pooled within-group size component, was adopted by Thorpe et al. (1982) and is explained in Thorpe (1983a, 1983b).

Data sets for morphometric purposes in ichthyology have generally been composed of traditional characters, namely total and standard length, total height, etc. The general biases presented by these traditional data sets, namely preponderance of some direction over others, dense coverage measurements in some areas of the body, and paucity elsewhere (Humphries et al. 1981), can be overcome by using the "Truss" network method (Strauss and Bookstein 1982). There is increasing evidence that the Truss method is much more powerful in describing morphological variation between closely related fish taxa

TABLE 3. Pooled within-group correlation coefficients between the 16 multiple group principal components (MGPC) and total length. Component 1 is the "size" vector; components 2–16 are "shape" vectors. Correlation exists only between the "size" vector and total length as measured from the specimens used.

| MGPC | Correlation coefficient |
|---|---|
| 1 | −0.825 |
| 2 | 0.103 |
| 3 | 0.051 |
| 4 | −0.051 |
| 5 | −0.037 |
| 6 | 0.022 |
| 7 | −0.045 |
| 8 | 0.072 |
| 9 | 0.049 |
| 10 | 0.019 |
| 11 | −0.043 |
| 12 | 0.050 |
| 13 | 0.032 |
| 14 | −0.054 |
| 15 | −0.037 |
| 16 | −0.076 |

with respect to traditional measurements (Strauss and Bookstein 1982; Winans 1984; Cataudella et al. 1987).

## Materials and Methods

The six stocks used in this study were collected in wild and artificial Italian water bodies managed by private fish farmers. The localities and the origin, together with the squamation, number of individuals, and the average total length for each stock, are reported in Table 1. Stocks have been named with a three-letter code, i.e. from CCA to CCF. The origin of each stock is known except for CCA (Table 1); moreover, CCC, originating from the River Tiber, has undergone frequent and successive introductions.

Twenty-one distance characters have been recorded on X-ray pictures between homologous landmarks (Fig. 1) following the rapid and accurate Truss method adopted from Strauss and Bookstein (1982). The Truss network consists of a series of quadrilateral cells, each one defined by four landmarks among which are four edges and two diagonal distances (Fig. 1). Landmarks are true and unambiguous homologous points from specimen to specimen identified by anatomical features as indicated in Fig. 1. This procedure allows one to detect variation in linear dimensions in horizontal, vertical, and oblique directions throughout the entire body.

In contrast with univariate statistics, rules to test sample size adequacy in multivariate statistics are less known and most of them are asymptotic and inexact (Neff and Marcus 1980). Harris (1975) suggested that if the number of the individuals minus the number of the variables used is greater than 30, than the sample can be considered as large. In our case this criterion gives a value of 71, and so we assume that the number of specimens used in this study (Table 1) is adequate for our purposes. Random resampling, varying sample size and content, is one method of tackling this problem but is beyond the scope of this paper.

There is a bias in the age/size of fish in the various samples, some being predominantly young fish and others being biased towards older, larger fish (Table 1). Consequently, we wished to define a "size/growth" component and remove its influence

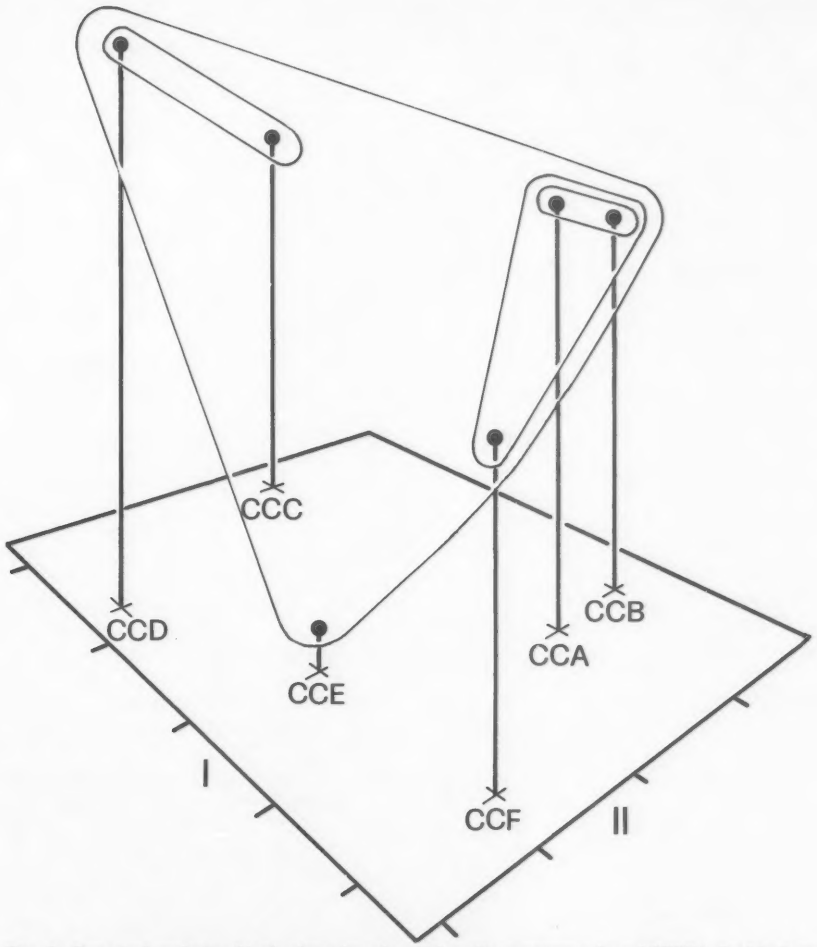


FIG. 2. Canonical analysis of body shape based on "shape" components from MGPCA. In this three-dimensional plot, the third canonical variate is the vertical axis. Units along canonical axes represent pooled within-group standard deviations. Progressive contour clustering between group centroids is determined by UPGMA computed on Mahalanobis distances. The close clustering of stocks with common geographic origin is evident, as is their distinctness from others as a consequence of long-term geographic isolation.

TABLE 4. Eigenvalues, canonical correlations, and percentage of variance of the first three canonical functions (CV) produced by "shape" CVA.

| | CV I | CV II | CV III |
|---|---|---|---|
| Eigenvalue | 2.63999 | 1.22893 | 0.93123 |
| Canonical correlation | 0.85163 | 0.74253 | 0.69440 |
| % variance | 45.429 | 21.147 | 16.025 |

from the data set. When there are several groups, as in this case, the use of simple principal component analysis to define and remove size is generally inappropriate because of the confusion of within- and between-group size differences. This is explained geometrically in Thorpe (1976, 1983a, 1983b). To overcome this problem, we used MGPCA, which extracts vectors from the pooled within-group covariance matrix. This gives a pooled within-group size vector when size is a component of the within-group variation due to growth effects. The requirements are as for canonical variate analysis (CVA; Thorpe 1983a), except that equality of variance along the growth axis is not required (Thorpe 1983a, 1983b).

To assess affinities between carp stocks, we used CVA. CVA orders groups along axes of maximum variation in such a way as to maximize among-group variance relative to within-group variance.

When all the component scores from MGPCA are subjected to CVA, this gives exactly the same results as a CVA on the raw data. When the component scores from the "size" vector are excluded, this gives a size-independent "shape" CVA. A scatter diagram based on the main canonical variates gives a visual portrayal of the relative similarity of the stocks.

## Results

CVAs performed separately on the two sexes did not reveal sexual dimorphism. Consequently, further analyses were performed on data pooled irrespective of sex.

TABLE 5. Above the diagonal: "shape" (upper row) and "size-dependent" (lower row) matrices of Mahalanobis distances between group centroids. Below the diagonal: matrix of $F$-values (upper row) and of Rogers' genetic distances (lower row) between all pairs of the six stocks. The degrees of freedom are 15 and 72; $P < 0.001$ except for the value marked with an asterisk which is not significant.

| | CCA | CCB | CCC | CCD | CCE | CCF |
|---|---|---|---|---|---|---|
| CCA | — | 1.80 | 3.56 | 3.65 | 4.61 | 2.92 |
| | | 2.26 | 3.70 | 4.63 | 5.70 | 3.47 |
| CCB | 1.39* | — | 3.28 | 4.09 | 4.45 | 3.25 |
| | 0.063 | | 4.05 | 5.87 | 6.48 | 4.59 |
| CCC | 7.63 | 5.28 | — | 3.16 | 4.69 | 4.33 |
| | 0.39 | 0.25 | | 3.64 | 5.23 | 4.41 |
| CCD | 5.61 | 6.08 | 4.83 | — | 4.78 | 4.22 |
| | 0.17 | 0.16 | 0.18 | | 4.81 | 4.33 |
| CCE | 4.63 | 3.99 | 5.18 | 4.61 | — | 5.25 |
| | 0.32 | 0.26 | 0.25 | 0.29 | | 4.92 |
| CCF | 4.03 | 4.23 | 10.52 | 7.11 | 4.68 | — |
| | 0.04 | 0.08 | 0.32 | 0.27 | 0.32 | |

TABLE 6. Classification matrix according to "shape" CVA. Note that the major incongruencies concern stock CCC from the River Tiber as a consequence of uncontrolled crosses with different carp stocks (see text and Table 1 for further details).

| | | Number of cases classified into group: | | | | | |
|---|---|---|---|---|---|---|---|
| Group | Percent correct | CCA | CCB | CCC | CCD | CCE | CCF |
| CCA | 77.8 | 14 | 3 | 0 | 1 | 0 | 0 |
| CCB | 61.5 | 4 | 8 | 0 | 0 | 0 | 1 |
| CCC | 63.6 | 1 | 2 | 21 | 3 | 3 | 3 |
| CCD | 100 | 0 | 0 | 0 | 13 | 0 | 0 |
| CCE | 100 | 0 | 0 | 0 | 0 | 5 | 0 |
| CCF | 87.5 | 2 | 0 | 0 | 0 | 0 | 14 |
| Total | 81.73 | | | | | | |

The within-group covariance matrices were computed from the 21 original characters for each stock independently and then pooled to give a pooled within-group covariance matrix. Since the determinant of this matrix equals zero, we carefully checked those characters that contribute least, i.e. characters expressing low variance. When these were excluded (characters between landmarks 2–3, 3–4, 3–5, 6–7, and 6–8; Fig. 1), the resulting pooled within-group covariance matrix was real, symmetric, and positive definite.

The MGPCA run on the 16 selected characters of all six groups produced a clear "size" vector (with coefficients of similar magnitude and sign) associated with the largest eigenvalue (Table 2). The remaining components were taken as representing "shape." To confirm this interpretation the pooled within-group correlation between the 16 multiple group principal components (MGPC) and an independent measure of size, i.e. total length, was computed. This alternative measure of size is highly correlated with the "size" component, i.e. MGPC 1, and is uncorrelated with the "shape" components, i.e. MGPC 2–16 (Table 3), thereby confirming this interpretation of the components.

Discriminant functions based on the score from the MGPCA "shape" components completely distinguish among the six stocks except CCA from CCB.

The separation of the stocks on the group means on the first three canonical variates is given in Fig. 2. The first, second, and third canonical variates absorb 45.429, 21.147, and 16.025% of the total dispersion, respectively, to explain cumulatively 82.601% of the total variation (Table 4). The significance level of the discrimination of the six stocks is shown by the values of the $F$-matrix (Table 5). The classification matrix shows a percent of correct classification varying from 100 to 61.5% (average 81.73%) (Table 6).

It might be argued that since the "size" component alone, as detected by MGPCA, absorbs 91.398% of total variance, it would have been meaningful to perform CVA on "shape" components explaining 8.602% only of total variance. However, it must be stressed that the contribution of the "size" component (as any other within-group component) to between-group discrimination bears no relationship to the percentage of within-group variance it expresses (Thorpe 1983a, 1983b). In this case, a parallel "size-dependent" CVA demonstrates that the "size" component expresses only 25.919% of the between-group discrimination, and in any event, growth bias between samples precludes its inclusion.

To summarize intergroup affinities, Mahalanobis (1936) distances between group centroids were computed (Table 5). These distances test the equality of group means for each pair of groups and can be used as the only realistic measure of multivariate distance (Reyment et al. 1981).

A UPGMA clustering (Sneath and Sokal 1973) computed on Mahalanobis distances between centroids (Fig. 2) has been superimposed on the three-dimensional plot of canonical scores to show the phenetic relationships between stocks (Fig. 2). The pattern of phenetic similarities (Fig. 2) shows a direct relationship with the geographic origin of each stock (Table 1), but not with their squamation (i.e. mirror, leather, or complete). The eastern European stocks (CCB, CCF) cluster first together with the stock of unknown origin (CCA). The isolated position of CCE with respect to the other groups reflects its origin. The stock CCE originated from Germany where carp culture was established long ago with selection and breeding programmes, and it has not been contaminated by later introductions since it was imported into Italy some 40 yr ago. CCC originates from a population of the River Tiber, and it has been subjected to repeated crosses with carps belonging to an Albanian stock originating from China. A genetic constitution with "Chinese" genes of CCD was speculated by some of us (Cataudella et al. 1987) and might account for its clustering with CCC.

## Discussion

A previous paper (Cataudella et al. 1987) reported the multilocus electrophoretic results carried out on the same individuals of the six stocks. Although no fixed different allele was found in 13 enzymatic systems (40 loci), the analysis of allele frequencies at polymorphic loci (Ca-1, Est-3, Est-4, Ldh-1, Pgm) did characterize the stocks to a certain degree. We computed a principal coordinate analysis (PCOA) on Rogers' (1972) genetic distances between the six stocks (Table 5). PCOA is an ordination technique which allows one to compute principal components from a distance matrix (Gower 1966). Figure 3 shows the UPGMA contour clustering, computed on Rogers' genetic distances, superimposed on the PCOA three-dimen-

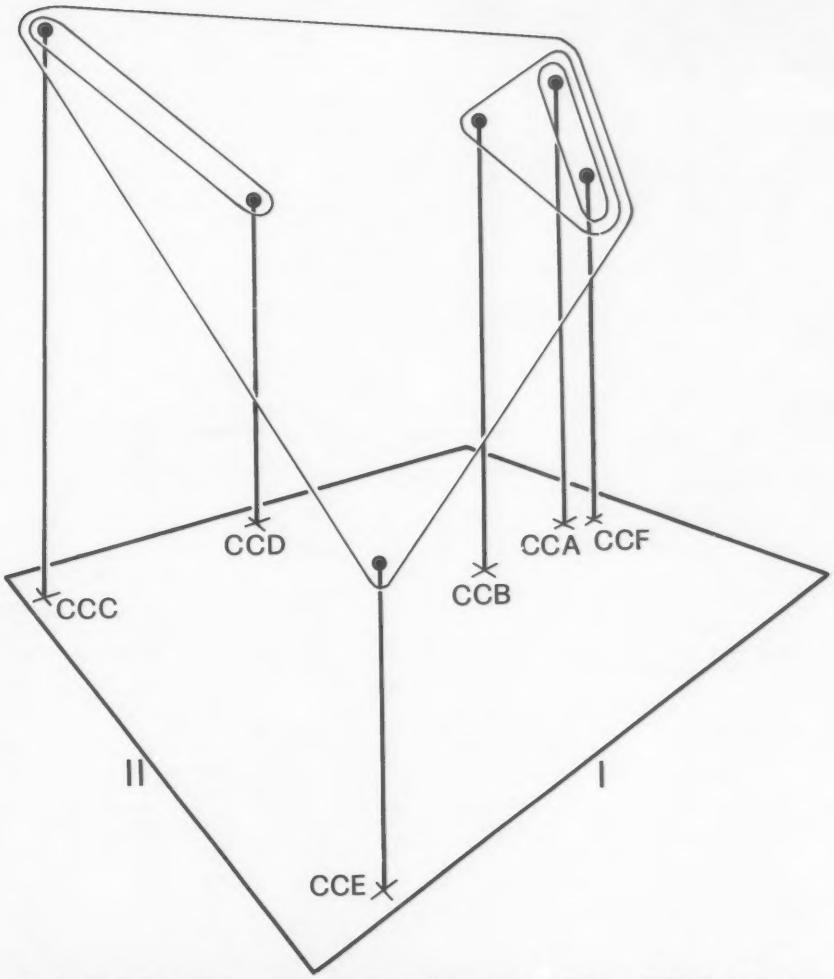


FIG. 3. Principal coordinate analysis computed on Rogers' genetic distances (Table 5). In this three-dimensional plot, the third principal component is the vertical axis. Progressive contour clustering between stocks is determined by UPGMA computed on Rogers' genetic distances. The pattern of genetic similarities between carp stocks is congruent with the one depicted from morphometric analyses (see Fig. 2).

sional scatter diagram. The general congruency in the distinction patterns from the "shape" morphometric (Fig. 2) and genetic (Fig. 3) analyses is evident; therefore, these two analyses corroborate one another. The only discrepancy between the genetic and morphometric relationships is the ordering of the clustering in the CCA, CCB, CCF set. However, the cophenetic correlation between Rogers' genetic distances and "shape" Mahalanobis morphometric distances (Table 5) is highly significant ($r = 0.704$, $P < 0.005$) in contrast with nonsignificant cophenetic correlation ($r = 0.376$) between genetic and "size-dependent" Mahalanobis distances (Table 5) as detected by the parallel CVA performed on "size" plus "shape" components. This evidence suggests that our "shape" analysis reflects a genetic pattern of variation between stocks and that this variation apparently is not strongly influenced by local pond environmental factors.

These findings show that the incongruencies previously found between morphometric and genetic results (Cataudella et al. 1987) are a consequence of the bias in growth within the samples. Although nothing is known of the heritability of these morphometric traits, it is evident that the "shape" character analysis provided by MGPCA depicts a pattern of morphometric variation which is consistent with the "genetic" constitution of the stocks.

Congruence between allozymic and morphometric data sets has been often a source of debate (Lewontin 1984). The frequent lack of congruence is generally attributed to different evolutionary forces operating on the two sorts of characters and to differences in the descriptive power of statistical tests employed in the analysis of morphometric and allozymic data sets. Although a strict congruence is not a priori to be found, our results indicate that the stocks examined are morphometrically distinguishable and that their relationships reflect their geographic origins as well as their genetic constitutions. We suggest that multivariate morphometry represents a powerful and convenient tool for carp stock identification and monitoring in

aquaculture. Aquaculture requires increasing contributions from basic research to accelerate its development on a scientific basis rather than through trial and error.

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# Energetic Constraints of Juvenile Chum Salmon (*Oncorhynchus keta*) Migrating in Estuaries[1]

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Wissmar, R. C., and C. A. Simenstad. 1988. Energetic constraints of juvenile chum salmon (*Oncorhynchus keta*) migrating in estuaries. Can. J. Fish. Aquat. Sci. 45: 1555–1560.

Experiments on fish feeding behavior, field measures of fish sizes, migration rate, and prey resources, and an energetic growth model are used to evaluate the growth of juvenile chum salmon (*Oncorhynchus keta*) during outmigration in an estuary. Fish growth is defined as the difference between energy intake and metabolic costs. Energy intake is estimated from prey densities and foraging parameters, namely pursuit swimming speeds and energetic expenditures, and prey handling and encounter rates. Relationships for foraging parameters as functions of fish sizes and field prey abundance are used to evaluate energy intake by fish in the estuary. This information and calculations of metabolic costs allow predictions of fish growth during the migration. Results show that metabolic costs of maintenance and migration lie in a delicate balance with food intake and growth. Growth efficiencies exceeding 20% occur when metabolic expenditures decline and energy intake rates permit growth without depletion of the food supply. Defining such energetic balances facilitates characterization of carrying capacities of coastal ecosystems and has implications for enhancement of juvenile fish growth and, in turn, fish survival and production at sea.

Des expériences sur le comportement alimentaire des poissons, des mesures sur le terrain de la taille des poissons, leur taux de migration et les proies, et un modèle énergétique de croissance sont des éléments utilisés pour évaluer la croissance de juvéniles de saumon kéta (*Oncorhynchus keta*) pendant la dévalaison dans un estuaire. La croissance des poissons est définie comme la différence entre l'apport énergétique et les dépenses métaboliques. L'apport énergétique est évalué à partir de la densité des proies et des paramètres relatifs à la recherche de nourriture, notamment la vitesse de nage pour poursuivre les proies et les dépenses énergétiques, ainsi que la manipulation des proies et le taux de rencontre. Les rapports pour les paramètres relatifs à la recherche de nourriture en tant que fonctions de la taille des poissons et de l'abondance des proies dans le milieu sont utilisés pour évaluer l'apport d'énergie des poissons dans l'estuaire. Ces données sur les dépenses métaboliques et leurs calculs permettent de prévoir la croissance des poissons pendant la migration. Les résultats montrent que les dépenses métaboliques our leur survie et leur migration sont en équilibre délicat avec l'apport alimentaire et la croissance. On observe une efficacité dépassant 20 % pour la croissance lorsque les dépenses métaboliques diminuent et que le taux d'apport énergétique permet la croissance sans épuisement des aliments. Définir de tels équilibres énergétiques facilite la caractérisation de la charge limite des écosystèmes côtiers et permet d'intervenir au niveau de la croissance des juvéniles, ce qui aura par la suite des incidences sur la survie des poissons et leur production en mer.

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Knowledge of bioenergetics of food intake and metabolic costs is necessary to understand the trophic dynamics of fish. However, such information is mostly limited to laboratory measurements of metabolic rates with little examination of natural situations (Ware 1978; Brett and Groves 1979; Brett 1986). An energetic perspective is especially important to our understanding of juvenile fishes, energy intake, metabolic costs, and growth while migrating in estuaries and nearshore marine waters (Van Winkle 1977; Simenstad et al. 1982; Simenstad and Wissmar 1984; Nichols et al. 1986). For example, high growth and increase in body size for various juvenile salmon in estuaries may offset high predation mortalities during the first few weeks at sea (Parker 1971; Healey 1982; Furnell and Brett 1986).

This paper presents an evaluation of the energetics of juvenile chum salmon (*Oncorhynchus keta*) during spring outmigration in an estuary. The objective of this work is to provide a basis for future energetic studies at the fish population level. We first give results of experimental determinations of foraging parameters for fish burst swimming speeds, prey handling times, and encounter rates as functions of different-sized fish preying on harpacticoid copepods. This information is used in a foraging paradigm to estimate the energetic values of food intake rates. We then incorporate this foraging concept into an energy mass balance model to examine food intake rates, metabolic costs, and amounts of energy left for growth during the spring outmigration. This energetic approach uses field data on prey standing crops, fish migration rates, and sizes.

Recently, it has become evident that carrying capacity limitations may exist for juvenile chum salmon in estuaries (Simen-

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stad et al. 1980; Simenstad and Salo 1980; Simenstad et al. 1982). These studies suggest that juvenile chums might have low food consumption and growth when they are most abundant. These conditions can coincide with declines in populations of the juvenile chum salmonid's principal prey organism, harpacticoid copepods (Healey 1979; Sibert 1979; Simenstad et al. 1980; Simenstad and Salo 1980). As in most habitats, it has been difficult to predict how growth of juvenile salmon in estuaries is affected by overexploitation of food supplies. The problem primarily is that little is known of the bioenergetic requirements of fish in nature. This study begins to address these shortcomings by examining the energetics of juvenile chum food intake, metabolic costs, and the amounts of energy left for growth during migration in an estuary. Such information could be invaluable for investigation of potentially severe food-limiting situations for juvenile salmon, for instance, when fish densities increase at varying times through enhancement efforts.

## Methods

The net energy intake is described as

$$(1)\quad I/t = \frac{Y(Ai - T_f H)}{1 + YH}$$

where $Y$ = individual prey encounter rate (number per second), $A$ = assimilated fraction of energy ingested, $i$ = energetic content (joules) of preferred copepod prey, $T_f$ = energetic expenditure of burst swimming while feeding (joules per second), and $H$ = handling times (seconds) for a juvenile fish to pursue and consume a prey item (Werner and Hall 1974; Mittelbach 1981). Equation (1) differs from formulations for other fishes (Werner and Hall 1974) in that $T_f$ and $H$, for juvenile chum salmon, represent constant swimming behavior while foraging and minimal time in manipulating prey after capture.

$T_f$ is described as

$$(2)\quad T_f = a_0 W^{0.44} U_f^{2.42}$$

where $a_0$ = intercept of the metabolic cost - body weight relationship ($1.94 \times 10^{-7}$) and $U_f$ = the swimming speed (centimetres per second) (Brett and Glass 1973; Ware 1975, 1978). Equation (2) and power values are summarized in Ware (1978). In this study, metabolic costs during burst swimming speeds ($U_f$) while feeding are assumed to differ little from the metabolic cost – body weight relationship for migration costs. This assumption was made because the metabolic cost of burst swimming has not been determined empirically for juvenile chum salmon. It draws upon similarities in relationships of oxygen consumption as a function of swimming speed for similar-sized juvenile sockeye (*O. nerka*) and coho salmon (*O. kisutch*) (Averett 1969; Brett and Glass 1973; Brett 1983; Puckett and Dill 1984). We recognize that oxygen consumption as a metabolic indicator may give minimal estimates of the costs of burst swimming (Puckett and Dill 1984). Also, burst or fast-acceleration swimming is thought to be very inefficient (Webb 1982).

$Y$, $U_f$, and $H$ were estimated in aquaria experiments. Aquaria (35 L) studies were conducted under controlled light intensities (31 lx) and temperatures (9 ± 1°C) at the University of Washington's Friday Harbor Laboratory, Friday Harbor, Washington. We used experienced fish to reduce variability introduced by naive fish (Werner et al. 1981). Fish were conditioned by feeding upon harpacticoid prey prior to experimental trials. Fish from 37 to 72 mm (fork length) in size were fed adult harpacticoid copepods (*Tigriopus californicus*) obtained from tidal pools and maintained in the laboratory at the above light and temperature conditions. These prey approached the sizes of the most common harpacticoid prey (~0.2 mm in length) in natural diets (Simenstad et al. 1980).

Video recordings of fish in aquaria were used to examine prey encounter rates ($Y$) and handling times ($H$) as functions of prey density and fish size and burst swimming speeds ($U_f$) for different-sized fish. Relationships from those fish feeding experiments and prey density were used to estimate energy intake. Energy conversions for all calculations in this study were after Crisp (1971) and Elliot and Davidson (1975). Energy contents were estimated by converting fish and prey wet weights to dry weights and then multiplying by joules, where 4.184 J = 1 cal or 1 mg dry wt.

The energetics of juvenile chum salmon during spring outmigration are followed by evaluating the energetic constraints that determine the amount of energy left for growth. We define the energy left for fish growth (joules per second) as the difference between net energy intake (eq. (1)) and expenditures of standard metabolism and migration swimming (Winberg 1956; Ware 1975). Growth ($G$) is described as

$$(3)\quad G = I/t - (T_s + T_m)$$

where $I/t$ = net energy intake (eq. (1)), $T_s$ = standard metabolism, and $T_m$ = migration swimming cost. $T_s$ (Ware 1978) is given by

$$(4)\quad T_s = a_1 W^{0.8}$$

where $a_1$ = intercept of the metabolism – body weight relationship ($2.03 \times 10^{-4}$) to the power 0.8. $T_m$ is defined as

$$(5)\quad T_m = a_0 W^{0.44} U_m^{2.42}$$

where $a_0$ = intercept of the migration metabolic cost – body weight relationship ($1.94 \times 10^{-7}$) and $U_m$ = migration swimming speed (centimetres per second) (Ware 1978).

Field data used in the study of fish energetics during outmigration include juvenile chum salmon lengths, weights, and migration rates, harpacticoid copepod standing stocks, and water temperatures (Salo et al. 1980; Simenstad et al. 1980; Bax 1982, 1983). Data were collected from sublittoral waters of Hood Canal, a fjord in Puget Sound, Washington. Temperature corrections for metabolic calculations were after Winberg (1956). Water temperatures ranged in the field from 8 to 11°C.

## Results and Discussion

### Feeding Behavior Experiments

Average burst swimming speeds ($U_f$) and calculated energy expenditures ($T_f$) while feeding upon adult harpacticoid copepods in aquaria increased with fish size (Fig. 1A). The effect of fish size was most pronounced for energy expenditures because of the exponential metabolism body weight – swimming speed relationships (eq. (2)). For example, in 145 trials of fish ranging from 60 to 63 mm in length and with a mean pursuit speed of 8 $cm \cdot s^{-1}$, the fish displayed an energy expenditure of $18 \times 10^{-5}$ $J \cdot s^{-1}$ or an increased cost 11-fold greater than for 37-mm fish. These energy expenditures compare well with average metabolic costs for burst swimming by similar-sized juvenile coho salmon (Puckett and Dill 1984).

Average times for different-sized fish to handle prey ($H$) indicated that handling times (mean $H$ of 31–73 trials for

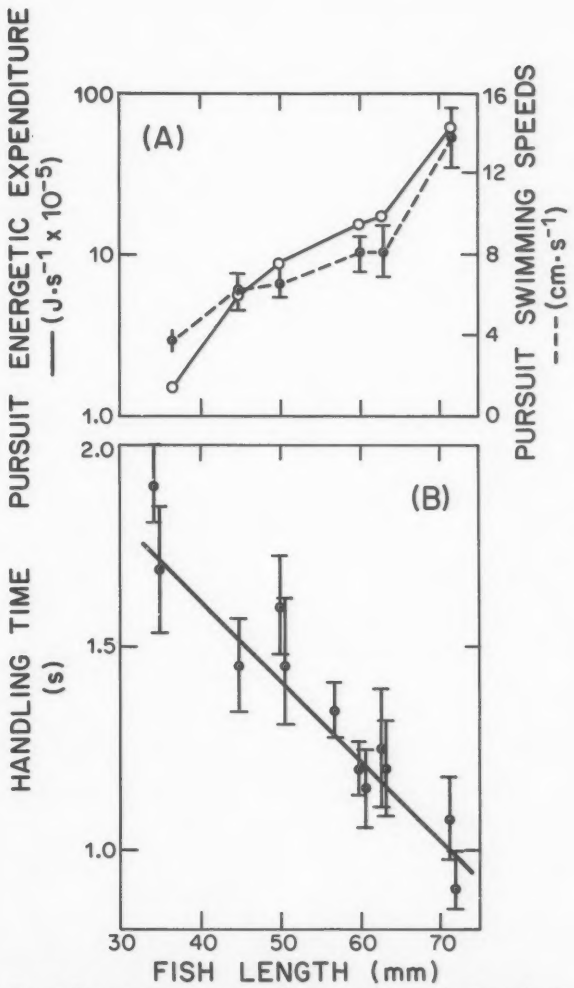


FIG. 1. Foraging parameters for different sizes of juvenile chum salmon feeding on adult harpacticoid copepods. (A) Mean burst swimming speeds (±1 SE) and energy expenditures, $T_f$; sample sizes for mean burst swimming speeds ($U_f$) ranged from 31 to 88. (B) Mean prey handling times (±1 SE); regression ($Y = 2.47 - 0.02X$, $r^2 = 0.77$, $n = 11$) significant at $P = 0.10$; sample size for mean handling ranged from 31 to 73.

different-sized fish) decreased significantly with increasing fish size ($Y = 2.47 - 0.02X$, $r^2 = 0.77$, $P = 0.10$). Mean handling times ranged from 0.9 to 1.8 s (Fig. 1B).

Prey encounter rates ($Y$) for all fish sizes ranged from 0.02 to 1.40 copepods·s$^{-1}$ and were directly proportional to prey densities (Fig. 2). Low encounter rates of <0.1·s$^{-1}$ were common for small fish (<60 mm) which fed upon inactive and low-density prey (<4.5·L$^{-1}$) on aquaria bottoms. This bottom or epibenthic feeding behavior by small fish has been noted in nature for juvenile chum salmon and other fishes (Werner and Hall 1974; Mittelbach 1981; Simenstad and Wissmar 1984). The lower encounter rates appeared related to prey inactivity, which reduced cueing by the fish. The highest encounter rates, 0.6–1.4·s$^{-1}$, were observed for larger fish (>60 mm) in the water column. Increases in $Y$ coincided with greater prey

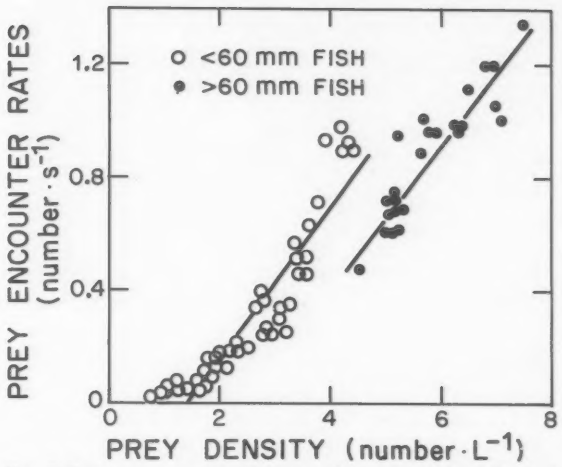


FIG. 2. Prey encounter rates, $Y$, by two size groups of juvenile chum salmon in relation to adult harpacticoid copepod densities. Regressions for <60 mm fish ($Y = -0.38 + 0.26X$, $r^2 = 0.83$, $n = 49$) and >60 mm fish ($Y = -0.61 + 0.25X$, $r^2 = 0.81$, $n = 26$) significant at $P = 0.05$.

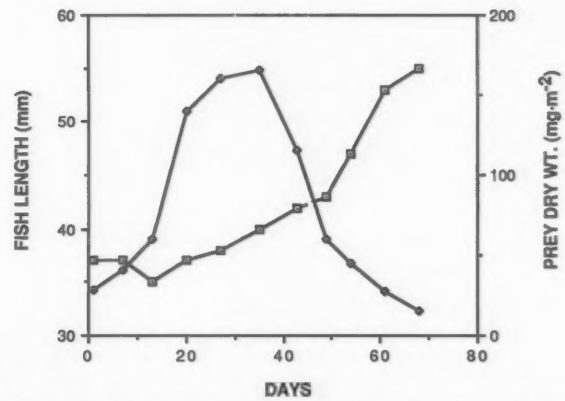


FIG. 3. Juvenile chum salmon sizes (open squares) and harpacticoid prey standing crops (solid squares) during the spring outmigration period (after Simenstad et al. 1980; Salo et al. 1980).

TABLE 1. Range of migration rates for juvenile chum salmon during spring in Hood Canal, Washington, 1977–79 (after Bax 1982).

| Month | Migration rate ranges (km·d$^{-1}$) |
|---|---|
| February | 4–12 |
| March | 4–14 |
| April | 7–9 |
| May–June | 4 |

activity and densities. These conditions appeared to motivate fish to increase burst swimming behavior. In turn, faster swimming speeds of the larger fish minimized the evasive ability of prey.

## Field Data

Juvenile chum salmon sizes and harpacticoid prey standing crops during the spring outmigration period are shown in Fig. 3.

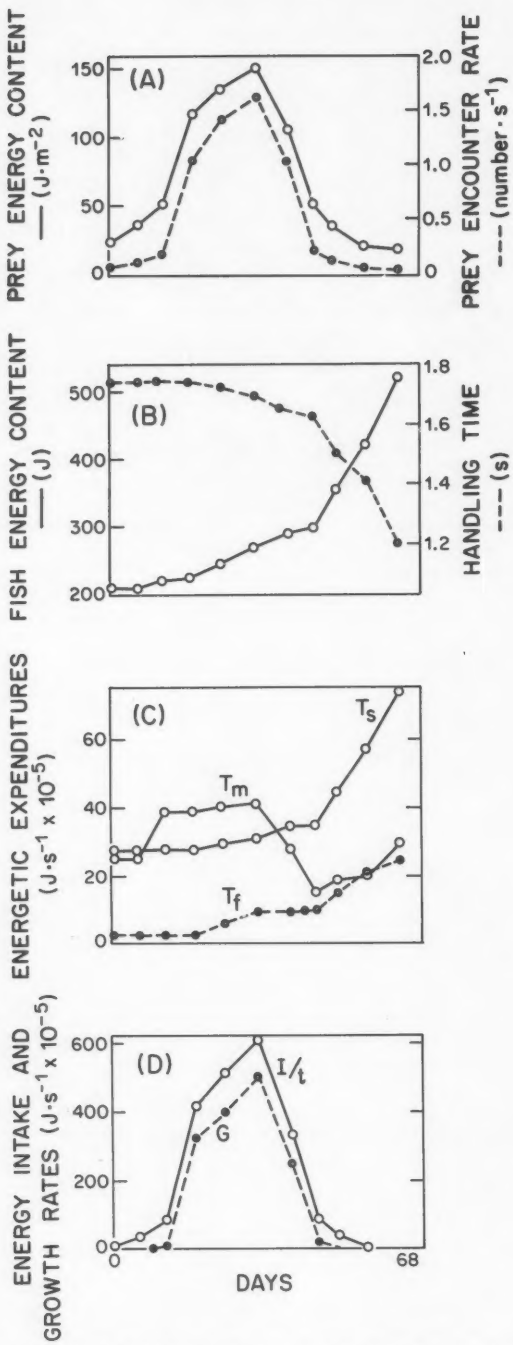


FIG. 4. Energetic and behavioral components for juvenile chum salmon during the estuarine migration. (A) Mean energy contents for adult harpacticoid copepod prey and prey encounter rates; (B) mean energy contents for fish and handling times; (C) energetic expenditures of standard metabolism ($T_s$), burst swimming ($T_f$), and migration swimming ($T_m$); (D) net energy intake ($I/t$) and growth rates ($G$).

During the early run of February and March, fish range in size from 35 to 40 mm with a mean weight of about 0.5 g. The fish usually occupy sublittoral seagrass beds with residence times approaching about 1 wk (Simenstad et al. 1980; Simenstad et al. 1982; Salo et al. 1980). Migration rates >12 km·d$^{-1}$ (Table 1) can coincide with sparse prey levels (Fig. 3). These relatively high migration rates have been attributed to wide-ranging fish searching for more adequate forage conditions and to influences of rapid surface currents that may transport fish (Simenstad and Salo 1980; Simenstad et al. 1982; Bax 1982, 1983). Later in April–May, as fish become larger (~55–60 mm or 1.2–1.5 g), migration rates decline.

## Energetics of Juvenile Chum Salmon during Spring Outmigration

The above experimental relationships for $U_f$, $H$, and $Y$ (Fig. 1 and 2) and field data for fish and prey (Fig. 3; Table 1) were used in eq. (1)–(4) to estimate fish energetics of food intake, metabolic costs, and growth during outmigration (Fig. 4A–4D). The energy mass balance approach (eq. (3)) permits an evaluation of the energetic constraints that determine the amount of energy left for growth during the spring outmigration period.

Our estimates of prey encounter rates ($Y$) for the migration period follow the pattern of prey concentrations, with the highest $Y$ values occurring in March. The lowest were in the early (February) and late phases of the migration period (Fig. 4A). Prey energy levels, like prey densities, peaked (117–150 J·m$^{-2}$) in early March and then declined throughout the remainder of the migration.

Fish handling times ($H$) for prey during the migration period declined with increasing fish sizes. The lowest $H$ times were for larger fish (523 J) and approached 1.2 s (Fig. 4B). The respective energy contents of fish throughout the migration period ranged from 209 to 523 J·fish$^{-1}$ (Fig. 4B).

During the outmigration, burst energetic costs for juvenile chums ($T_f$) increased with fish size (Fig. 4C). $T_f$ costs ranged from $3 \times 10^{-5}$ to $24 \times 10^{-5}$ J·s$^{-1}$, with the highest occurring at the end of the migration. For the entire migration period, $T_f$ averaged 11% of the total energy cost ($\Sigma T = T_f + T_s + T_m$). We suggest that low $T_f$ costs relative to other metabolic expenditures indicate an energetically efficient burst behavior by these fish when feeding upon harpacticoid prey.

The major metabolic costs to juvenile chum were due to migration swimming ($T_m$) and standard or maintenance metabolism ($T_s$). For the entire migration period, $T_m$ and $T_s$ were 42 ± 15% and 47 ± 8% of total costs ($\Sigma T$), respectively. The highest $T_m$ costs, $25 \times 10^{-5}$ to $41 \times 10^{-5}$ J·s$^{-1}$, appeared during the early and midphases of the migration, when swimming speeds ranged from 10 to 12 cm·s$^{-1}$ (Fig. 4C). $T_m$ costs declined in the late phase, when swimming speeds averaged 8 cm·s$^{-1}$. The relatively constant $T_m$ expenditure for the migration period, ± 15%, may signify fish behavior that favors energy efficiency in terms of being adapted to search for prey during daily cruising.

$T_m$ plus $T_s$ metabolic costs appeared most critical for fish when prey resource levels were low (Fig. 4A, 4C, and 4D). During the lowest prey levels at the beginning and end of the migration, total $T_m$ and $T_s$ costs either approached or exceeded energy intake ($I/t$). As a result, growth was limited. $T_s$ costs were especially significant during the late phase, when they averaged 58% of $\Sigma T$. $T_s$ percentages exceeding 50% of $\Sigma T$ for juvenile salmon have been suggested as being common when food becomes scarce (Ware 1978).

TABLE 2. Comparison of daily growth rates of this study with values for juvenile chum salmon from the literature. Fish weight, daily growth, daily rations, and temperature conditions are indicated for various sources. Fixed rations used by LeBrasseur (1969) and Volk et al. (1984) consisted of natural prey taxa. Other sources include wild rations (Healey 1979) or natural prey populations.

| Wet wt. (g) | | Growth rate ($J \cdot d^{-1}$) | | Growth rate (% $bw \cdot d^{-1}$) | | Daily ration (% $bw \cdot d^{-1}$) | | Temp. (°C) | Source |
|---|---|---|---|---|---|---|---|---|---|
| Mean | Range | Mean | Range | Mean | Range | Mean | Range | | |
| 0.68 | 0.50–1.30 | 24[a] | 1–38 | | | 20.5 | | 9–11 | This study |
| 0.66 | 0.35–1.20 | 17[b] | | 15.0 | | 15.0 | | 10–15 | Healey 1979 |
| 1.10 | 0.60–4.50 | | 0–64[b] | | 0–3.4 | | 1.0–19.0 | 8–10 | Volk et al. 1984 |
| 2.91 | 0.45–9.90 | 51 | 4–249 | 4.2 | 2.0–6.0 | | 3.0–17.0 | 14–16 | LeBrasseur 1969 |

[a]Growth ($J \cdot d^{-1}$) was calculated using the mean daily ration of 20.5% bw for juvenile chum in our study area (Simenstad et al. 1980), the average energy content for fish body weight (251 J), and from our model estimate of gross growth efficiency (46%). Energy content was estimated by converting fish body wet weight to dry weight and then multiplying by joules, where 4.184 J = 1 cal or 1 mg dry wt. (Crisp 1971). Accordingly, a daily ration of 60 J times the mean growth efficiency yields a mean daily growth rate of 24 $J \cdot d^{-1}$.

[b]Growth ($J \cdot d^{-1}$) was estimated by converting fish body weight to joules (see footnote a) and multiplying by the growth rate (% $bw \cdot d^{-1}$).

The impacts of $T_m$ and $T_s$ costs on fish metabolism were greatly reduced when prey were abundant during the midphase of the migration (Fig. 4A, 4C, and 4D). The energy balance for the fish during this phase showed that energy intake (70 to 608 $J \cdot s^{-1}$) exceeded expenditures, $T_m$ and $T_s$ (59 to 82 $J \cdot s^{-1}$). Such an energetic condition appears to be of critical importance to the fish because it permits the allocation of energy to growth (7 to 536 $J \cdot s^{-1}$). These results suggest that at this stage of the migration the amount of energy left for fish growth appears to be directly dependent on higher levels of prey standing stocks in the estuary.

### Growth Efficiencies and Daily Growth Rates during Outmigration

Our evaluation of juvenile chum energetics during the spring outmigration showed that when growth occurred, growth relative to the energy of food consumed ranged from 3 to 68%. This gross growth efficiency averaged 46% and compared well with values of 40 to 55% for other juvenile salmon (Paloheimo and Dickie 1966: Ware 1978).

The mean gross growth efficiency, along with fish weight and daily food ration, was used to estimate the mean daily growth rate during the outmigration (Table 2). Our estimate of the mean daily growth for chum during the outmigration of 24 $J \cdot d^{-1}$ compared well with a mean growth rate of 17 $J \cdot d^{-1}$ (6.1% body weight (bw)$\cdot d^{-1}$) for juvenile chum salmon feeding on wild or natural prey in a British Columbia estuary (Healey 1979). Lower growth rates for small chum (<0.60 g) feeding on low levels of fixed rations consisting of natural prey taxa (<3% $bw \cdot d^{-1}$) have been shown not to exceed 4 $J \cdot d^{-1}$ or 2% $bw \cdot d^{-1}$ (LeBrasseur 1969; Volk et al. 1984) (Table 2).

## Conclusion

This study suggests that at gross growth efficiencies exceeding 40%, favorable foraging conditions exist in estuaries. At efficiencies less than 40%, fish growth and prey levels are low. We surmise that at efficiencies exceeding 40%, fish growth and prey levels are high enough that metabolic expenditures by the fish decline relative to energy intake rates. Under these conditions, the energy balance permits growth to occur without a marked depletion of the food supply. Clearly, if these insights hold upon further investigation at the fish population level, they should facilitate the definition of the carrying capacities for estuaries and other aquatic ecosystems in terms of prey and fish standing stocks and energetics.

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# Lake Acidification and Fisheries Project: Adult Brook Trout (*Salvelinus fontinalis*)[1]

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The following set of nine papers is the first of several planned for publication in the *Canadian Journal of Fisheries and Aquatic Science* (CJFAS) from the Lake Acidification and Fisheries (LAF) Project. These nine papers present toxicology, physiology, and histopathology results on adult brook trout (*Salvelinus fontinalis*) exposed in the laboratory to water quality conditions typical of acidified lakes and streams, with lowered pH, elevated aluminum concentrations, and low calcium concentrations.

## Project Overview

The overall goal of the LAF Project is to determine cause–effect relationships between surface water acidification and fish population responses by using bioassay data from the laboratory along with water quality and fish population data from the field, with all information integrated in a modeling framework. The specific steps have been to (1) establish extensive laboratory data sets on toxicological (survival, growth, reproduction) effects of combinations of acid, aluminum, and calcium concentrations on four fish species, brook trout, rainbow trout (*Salmo gairdneri*), white sucker (*Catostomus commersoni*), and smallmouth bass (*Micropterus dolomieui*), (2) determine for these same species the physiological/pathological mechanisms of acid and aluminum toxicity in soft water, to form a basis for understanding differences in species sensitivity and, thus, a basis for interpolating within the four-species data set to yet other fish species of intermediate sensitivity, and (3) develop, calibrate, and apply a computer modeling framework that uses the laboratory toxicity data sets, calibrated with water quality and population data from the field, to reliably estimate the probability of presence of fish populations under observed or projected water quality conditions in acidified or sensitive water bodies. This LAF modeling framework, described earlier by Van Winkle et al. (1986) and Christensen et al. (1988), goes beyond previous fish population modeling efforts related to acidification in its incorporation of extensive laboratory bioassay results and relevant life history information from the field. Some important applications of the LAF modeling framework include regional predictions of fish population status, given predicted trends in acidification, and estimates of fish population responses expected from fisheries management practices such as changes in stocking rates or fishing pressure.

Although the final product from the LAF Project research will be a set of models and a modeling framework for predicting presence or absence of fish populations, the extensive data sets on toxicology, physiology, and histopathology should be of immediate value to researchers and decision-makers and, as noted above, these results are being presented in a series of publications beginning with this issue of the CJFAS.

## Brook Trout Adults

In several related experiments, reproductively mature brook trout were exposed to as many as 27 different combinations of pH, aluminum, and calcium representative of acidic and acid-sensitive surface waters. Exposures continued for as long as 147 d and spanned the period from early vitellogenesis through spawning. Under extreme conditions (e.g. pH $< 5$, 486 μg Al/L, 0.5 mg Ca/L), significant effects were noted on survival, growth, and fecundity (Mount et al. 1988a), as well as plasma osmolality, sodium, estradiol, vitellogenin, and calcium (Mount et al. 1988b). But since the significant effects observed for reproductive parameters occurred only in combinations of acid, aluminum, and calcium that also affected survival and growth, Mount et al. conclude in these two papers that reproductive failure is not a likely explanation for recruitment failure in brook trout populations affected by acidification. Rather, these findings, along with results from experiments on brook trout early life stages (Ingersoll et al. 1988), lead to a conclusion that the mechanism of brook trout population loss in acidic surface waters is direct mortality of sensitive life stages.

In a series of experiments using the same ranges of acid, aluminum, and calcium concentrations used by Mount et al. (1988a), McDonald, Wood, and colleagues explored ionoregulatory, respiratory, and cardiovascular mechanisms of acid/aluminum toxicity in adult brook trout. They conclude that the primary mechanism of toxicity appears to be disturbance of normal ion regulation at the gill (Booth et al. 1988; McDonald and Milligan 1988; Wood et al. 1988a), although respiratory impairment may play a role under certain exposure conditions, especially higher calcium concentrations in the presence of low

[1]Special Editor for this collection of papers was Dr. P. E. K. Symons.

pH and elevated aluminum (Wood et al. 1988a; Walker et al. 1988). These conclusions were based on their findings that initial exposure to low pH causes massive losses of plasma electrolytes due to increased rates of ion loss coupled with inhibition of ion uptake. Adding aluminum to low pH exposures exacerbates this ionoregulatory disturbance. But adult brook trout that had been acclimated for 10 wk to sublethal concentrations of acid plus aluminum were resistant to subsequent challenges with elevated aluminum (McDonald and Milligan 1988; Wood et al. 1988b; Wood et al. 1988c). Tietge et al. (1988) also observed histological changes in gills of brook trout, such as increased volume density of chloride cells and increased diffusion distance, that were consistent with the reported ionoregulatory and respiratory effects of acid/aluminum exposure.

## The Future

Additional publications on completed and on-going research in the LAF Project include toxicology, physiology, and histopathology results from experiments with brook trout early life stages, results of similar experiments with critical life stages of rainbow trout, white sucker, and smallmouth bass, demonstrations and applications of the models and modeling framework built upon these results, and comparisons and syntheses covering results from all four fish species. A set of papers describing results with the brook trout early life stages is currently in review at CJFAS with anticipated publication in a forthcoming issue. Final revisions were recently completed on the LAF modeling framework for brook trout, and the LAF framework is now being used for several tasks in the National Acid Precipitation Assessment Program in the United States. Project staff are now completing toxicology and physiology experiments with rainbow trout, white sucker, and smallmouth bass, and as data from these experiments are compiled, they will be used to extend the LAF modeling framework for brook trout to these species.

## Data Availability

Because of the extensive nature of the toxicology data sets being produced in this project (e.g. daily observations on as many as 84 different exposure combinations of pH, aluminum, and calcium in 17 different experiments that together cover six life stages of brook trout), only summaries and applications of data are presented in the publications in this and anticipated issues of CJFAS. Because many investigators and agencies may find additional uses for the full toxicology data sets, they will be made available on computer disc or tape from the Electric Power Software Center, 1930 Hiline Drive, Dallas, TX 75207, USA.

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# Effects of Aluminum and Low pH on Net Ion Fluxes and Ion Balance in the Brook Trout (*Salvelinus fontinalis*)

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Booth, C. E., D. G. McDonald, B. P. Simons, and C. M. Wood. 1988. Effects of aluminum and low pH on net ion fluxes and ion balance in the brook trout (*Salvelinus fontinalis*). Can. J. Fish. Aquat. Sci. 45: 1563–1574.

Adult brook trout (*Salvelinus fontinalis*) were exposed for up to 11 d to one of a matrix of 18 Al, low pH, and $Ca^{2+}$ combinations, chosen as representive of acidified softwater environments in the wild. Reduction in water pH led to pH-dependent net losses of $Na^+$ and $Cl^-$ exacerbated by the presence of Al in the water and reduced by elevating $Ca^{2+}$. Any animal losing more than 4% of its total body $Na^+$ over the first 24 h of Al exposure had a greater than 90% likelihood of eventual mortality. $Na^+$ losses arose from inhibition of influx and stimulation of efflux. The inhibition was persistent and pH dependent. Addition of Al to acidified water had a slight further inhibitory effect on $Na^+$ influx and a large stimulatory effect on efflux. The latter was dependent on Al concentration, was the main cause of initial ion losses and mortality, and declined with time in surviving animals. All Al-exposed fish accumulated Al on their gills, but this was apparently mainly surface or subsurface bound, since no internal Al (plasma or liver) could be detected. Nonsurviving fish had substantially higher gill Al levels than survivors.

Pendant des périodes allant jusqu'à 11 d, on a exposé des ombles de fontaine (*Salvelinus fontinalis*) adultes à 18 différentes combinaisons de teneurs en Al et en $Ca^{2+}$ et de faibles pH, caractéristiques des conditions naturelles des eaux douces acides. Une réduction du pH de l'eau a mené à des pertes nettes de $Na^+$ et de $Cl^-$ liées au pH, aggravées par la présence d'Al dans l'eau et réduites par une augmentation de la teneur en $Ca^{2+}$. Les individus qui avaient perdu lus de 4 % de la teneur corporelle totale en $Na^+$ pendant les 24 premières heures d'exposition à l'Al étaient plus susceptibles de mourir dans plus de 90 % des cas. Les pertes de $Na^+$ proviennent de l'inhibition de son entrée, constante et dépendante du pH, et de la stimulation de sa sortie. L'apport d'Al aux eaux acides a un autre léger effet inhibiteur sur l'entrée du $Na^+$ et un grand effet stimulateur sur la sortie. En plus de dépendre de la teneur en Al, ce dernier effet est la principale cause des pertes initiales d'ions et de la mortalité et diminue en fonction du temps chez les individus survivants. Tous les poissons exposés à l'Al l'ont accumulé dans leurs ouïes mais il semble que cette liaison se soit surtout effectuée à la surface ou sous la surface, étant donné qu'aucune trace d'Al n'a été observée dans le plasma ou le foie. On a noté des teneurs plus élevées d'Al dans les ouïes des poissons morts que chez les survivants.

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The physiological responses of freshwater fish to environmental acidity have been studied extensively over the past 15 yr (see reviews by Fromm 1980; Wood and McDonald 1982; McDonald 1983b; Wood 1987). Considerable evidence from field and laboratory studies now points to ionoregulatory failure as the key factor leading to fish death in acidified waters, although the toxic effect may be exerted through disturbances to a host of physiological functions including fluid volume distribution, hematological and acid–base homeostasis, and oxygen uptake and transport (Packer 1979; Ultsch and Gros 1979; McDonald et al. 1980; Ultsch et al. 1981; Milligan and Wood 1982).

Two important environmental factors that influence the survival of fish in acidified water are water hardness and elevated concentrations of trace metals. Numerous studies have shown that increasing the concentration of external $Ca^{2+}$ (the major component of water hardness) enhances the survival of fish in acidified water. In fact, Wright and Snekvik (1978) showed, from a survey of 700 softwater lakes in southern Norway, that water $Ca^{2+}$ was at least as important as pH in determining fish population status. For acidic lakes and streams, there is mounting evidence that elevated concentrations of metals such as Al, Cd, Cu, Pb, Mn, Ni, and Zn, resulting from either atmospheric deposition or leaching of soils, may contribute to fish mortality (see reviews by Spry et al. 1981; Baker 1982; Campbell and Stokes 1985; McDonald et al. 1987). Of these metals, Al has caused the greatest concern because of its ubiquitous presence in soils and rocks and its widespread elevation in acidified waters. Indeed, Schofield and Trojnar (1980) found Al concentration to be the most important among 12 water quality parameters (more important than either pH or $Ca^{2+}$) in deter-

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TABLE 1. Chemical composition of water used to hold fish (Hamilton tap water) or acclimate fish (artificial soft water). For soft water the maximum ranges are reported. The large variation in [$Cl^-$] is the result of using $CaCl_2$ to make up the artificial soft water. Experimental water was prepared by adjusting pH of artificial soft water to pH 4.4, 4.8, or 5.2 and Al concentration to 111, 333, or 1000 μg/L. Over the course of an experiment, variations in experimental pH and Al were typically within 0.3 unit and 10%, respectively, of initial values.

| | Hamilton tap water (μequiv/L) | Artificial soft water (μequiv/L) |
|---|---|---|
| $Na^+$ | 540 | 34–50 |
| $Cl^-$ | 700 | 10–411 |
| $K^+$ | 20 | 2–3 |
| $Ca^{2+}$ | 2100 | 25 or 400 |
| pH | 7.8 | 6.3–6.6 |

mining brook trout (*Salvelinus fontinalis*) population status in 53 Adirondack lakes.

The chemistry of Al in natural waters is very complex; organic and inorganic ligands (e.g. humic acids, fluoride) play an important role in Al bioavailability, and water pH has a profound effect upon Al speciation and solubility (Driscoll et al. 1980; LaZerte 1984). Minimum Al solubility occurs at pH 6.0–7.0 and there is a greater than 7000-fold increase in solubility over the pH range from 6.0 to 4.0. At pH 4.0 the free aquo ion (i.e. $Al^{3+}$) is the predominant (>90%) monomeric species, while at pH 6.0, monovalent and divalent hydroxides predominate. Similarly complex is the toxicity of Al; several studies indicate that maximum toxicity occurs over the pH range 5.0–5.5 and decreases at lower and higher pH's (Freeman and Everhart 1971; Muniz and Leivestad 1980; Schofield and Trojnar 1980). Indeed, some authors have shown that low levels of Al can have a protective effect at pH's below about 4.4 (Muniz and Leivestad 1980; Schofield and Trojnar 1980; Baker and Schofield 1982).

Not surprisingly, given this complexity, much is still unknown concerning the mechanism of Al toxicity to fish. In general, the mechanism of Al toxicity appears in many respects to resemble that of $H^+$ toxicity; the main target of Al appears to be the gills (Schofield and Trojnar 1980; Muniz and Leivestad 1980; Buergel and Soltero 1983). Al accumulates on the gills in a concentration- and pH-dependent fashion (Neville 1985; Karlsson-Norrgren et al. 1986a, 1986b), thereby producing epithelial damage (Schofield and Trojnar 1980; Chevalier et al. 1985; Karlsson-Norrgren et al. 1986a, 1986b) and impairment of normal gill function (Staurnes et al. 1984; Witters 1986) resembling that seen with low pH alone (see McDonald 1983a for review). Furthermore, the physiological effects of Al appear to be similar to those of low pH in that ionic disturbances are prominent (Muniz and Leivestad 1980; Neville 1985) although there is some evidence to indicate that Al may also cause respiratory distress (Neville 1985). Finally, $Ca^{2+}$ appears to ameliorate Al toxicity, at least to juvenile fish, in much the same way as it does acid toxicity (Muniz and Leivestad 1980; Brown 1983).

To further clarify the mechanism of Al toxicity to fish, we have undertaken a study of the interactive effects of Al, pH, and $Ca^{2+}$ on adult brook trout. We were specifically interested in the following: the differences in toxic action of $H^+$ and Al, particularly in relation to their effects on branchial ion regulation and plasma ion balance; the nature of the protective effect of calcium, and the interrelations amongst ion losses, tissue accumulation of Al, and mortality. The levels of pH (4.4–5.2), Al concentration (0–1000 μg/L), and $Ca^{2+}$ concentration (0.5–8 mg/L, 25–400 μequiv/L) used in this study were chosen to represent a range of conditions likely to be encountered by brook trout populations in acidified softwater lakes in the northeastern United States and eastern Canada, a region where acid precipitation is considered to be a serious threat to aquatic ecosystems (Harvey et al. 1981; Schofield 1982; Kelso et al. 1986).

## Materials and Methods

### Experimental Animals

Adult brook trout of both sexes (100–300 g) were obtained from Goosen's Trout Farm in Otterville, Ontario. The fish were transported to McMaster University where they were maintained for at least 2 wk in flowing, dechlorinated Hamilton tap water (see Table 1) at 7–18°C. Prior to use, the fish were held in batches of 20–25 in 500-L green polyethylene tanks for 2–4 wk and acclimated to artificial soft water (Table 1) with a $Ca^{2+}$ concentration of either 25 or 400 μequiv/L (0.5 or 8.0 mg/L) at circumneutral pH (6.0–6.5) and a temperature of 10 ± 1°C. Soft water was prepared by adding NaCl and either $Ca(NO_3)_2$ or $CaCl_2$ (see Results) to deionized water obtained from a Culligan Aquacleer MP-500 reverse osmosis system. Water in the acclimation tanks was recirculated through a polyester wool/activated charcoal filter and was replaced at 2- to 4-d intervals. Fish in the holding tanks and the softwater acclimation facilities were fed commercial trout pellets every other day, but were not fed for 1 wk before an experiment.

### Experimental Protocol

Fish acclimated to soft water were placed individually in darkened Plexiglas flux chambers (McDonald and Rogano 1986) of nominal 2.7-L volume and supplied with flowing soft water (~0.5 $L \cdot kg^{-1} \cdot min^{-1}$) with the same levels of $Ca^{2+}$ and pH as in the acclimation tank. Water temperature in the flux chambers was maintained at 10 ± 1°C and continuous aeration ensured thorough mixing of the water. After a 24- to 48-h period of recovery from the stress of handling, fish ($N$ = 108) were exposed for 24 h to low-pH water (pH 4.4, 4.8, or 5.2) obtained by adding 0.1 N $H_2SO_4$ to 1500-L reservoirs of soft water supplying the flux chambers. Following this initial low-pH challenge, fish were then either maintained at the same pH for up to 10 d (controls, $N$ = 36) or were exposed to elevated concentrations of Al (111, 333, or 1000 μg/L) at the same pH ($N$ = 72). Al was added to the low-pH soft water as either $AlK(SO_4)_2$ or $AlCl_3$ (see Results). The chemical composition of the experimental water is given in Table 1. Water was never recirculated, but rather was run to waste after leaving the fish boxes.

The chemistry of aqueous Al is complex (Fig. 1). Water pH has profound effects on both Al speciation (Fig. 1A) and solubility (Fig. 1B), and even though the test concentrations of Al we employed were well below the solubility limits of Al in "new" solutions (see data points on Fig. 1B), the concentration of Al solutions tends to gradually decline with time as insoluble aluminum hydroxides form (Smith and Hem 1972). To avoid these speciation and solubility problems the following precautions were taken. First, batches of soft water containing Al (1500 L) were made up daily and used immediately. Consequently, Al-containing water did not "age" for more than 24–36 h. Second, the accumulation of waste products (e.g. ammonia) in the flux chambers was avoided by maintaining a

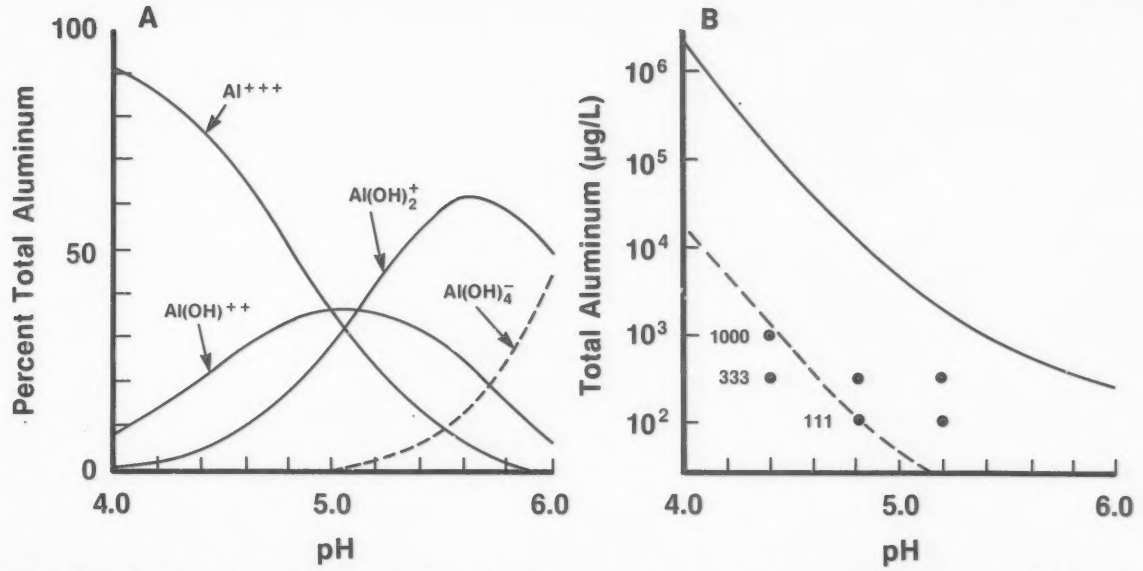


FIG. 1. Al chemistry in low ionic strength waters. Calculations based on thermodynamic equilibrium constants for 25°C and zero ionic strength from Johnson et al. (1981). (A) Al speciation diagram; relative concentrations of Al species at 25°C in freshwater. (B) Total soluble Al as a function of pH from the solution of amorphous $Al(OH)_3$ (solid line). Shown is the experimental matrix employed in this study (see Fig. 5). In natural waters, in equilibrium with gibbsite (crystalline $Al(OH)_3$), Al solubility would be as described by the broken line (Harvey et al. 1981).

continuous flow through the chambers and by discarding the water outflow. Third, the rates and pH of water flowing through the flux chambers were periodically adjusted so that the pH rose by less than 0.3 pH unit in transit through the chambers and the average of the inflow and outflow pH values equalled the desired experimental pH.

At the termination of an experiment the fish were killed by a sharp blow to the head, a terminal blood sample was obtained by caudal puncture, and the gills and liver were excised, blotted, and frozen, as was the remainder of the carcass, for later analysis of Al levels. Fish that died during an experiment were removed as soon as discovered and the gills and liver were excised as above. In addition, to establish control plasma ion levels, blood samples were taken from a group of fish acclimated for 18 d to soft water ($Ca^{2+}$ = 25 µequiv/L) at circumneutral pH.

## Ion and Ammonia Flux Measurements

Net exchanges of ions and ammonia between the animal and its environment were assessed by measuring the concentrations of $Na^+$, $Cl^-$, $Ca^{2+}$, $K^+$, and ammonia in water entering and leaving the flux chambers. At all sampling times (see Fig. 2 for sampling schedule), the total outflow of each chamber was collected for 2 min and the volume determined by weighing. Immediately afterward, a 150-mL sample of inflow was collected. About 60 mL of each sample was saved, preserved by addition of 20 µL of concentrated $HNO_3$ (analytical grade), and set aside for later analysis (see below). Net flux rates ($J_{net}$) were calculated from measured concentrations by the Fick principle:

$$J_{net}^{X}(\mu\text{equiv}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}) = \frac{(\text{input}[X] - \text{output}[X]) \times \text{water flow (L/min)}}{\text{fish mass (kg)}}$$

where $[X]$ is the concentration (microequivalents per litre) of substance $X$. Flux measurements were made at various times during the control period and during exposure to low pH and elevated Al. In some experiments, unidirectional $Na^+$ flux rates ($J_{in}$ and $J_{out}$) were measured by stopping water flow to and from the flux chambers, adding 1 µCi of $^{22}Na^+$ (New England Nuclear) to each chamber, and monitoring the disappearance of the radioisotope from the water over a 30-min period. $Na^+$ influx (microequivalents per kilogram per hour) was calculated according to the following formula:

$$J_{in}^{Na+} = \frac{(\ln Q_i^* - \ln Q_f^*)}{t \times W} \times Q_{out}$$

where $Q^*$ is the total amount of radioactivity in the medium at the beginning ($i$) and end ($f$) of the sampling interval ($t$), $Q_{out}$ is the average amount of $Na^+$ in the medium, $t$ is the sampling interval (hours), and $W$ is the body weight (kilograms) (after McDonald et al. 1983). $Na^+$ efflux ($J_{out}$) was calculated as the difference between $J_{in}$ and $J_{net}$. During these static flux measurements, water samples for ion and radiotracer analysis were taken from the flux chambers at 10-min intervals. At the same time the pH of the water was checked and adjusted to the desired level by the addition of either $H_2SO_4$ or KOH.

## Analytical Procedures

Concentrations of $Na^+$, $Ca^{2+}$, and $K^+$ in the water and blood plasma were measured with a Varian AA-1275 atomic absorption spectrophotometer after appropriate dilution. Water and plasma $Cl^-$ concentrations were measured by coulometric titration (Buchler–Cotlove chloridometer). Water ammonia concentration was determined by the salicylate–hypochlorite method of Verdouw et al. (1978). Al concentrations in the water, plasma, and tissues were measured on the Varian AA-1275 using an automated graphite furnace attachment (Varian GTA 95). Prior to analysis, tissues were digested in 2 volumes of concentrated $HNO_3$ and then diluted 1:105 with deionized

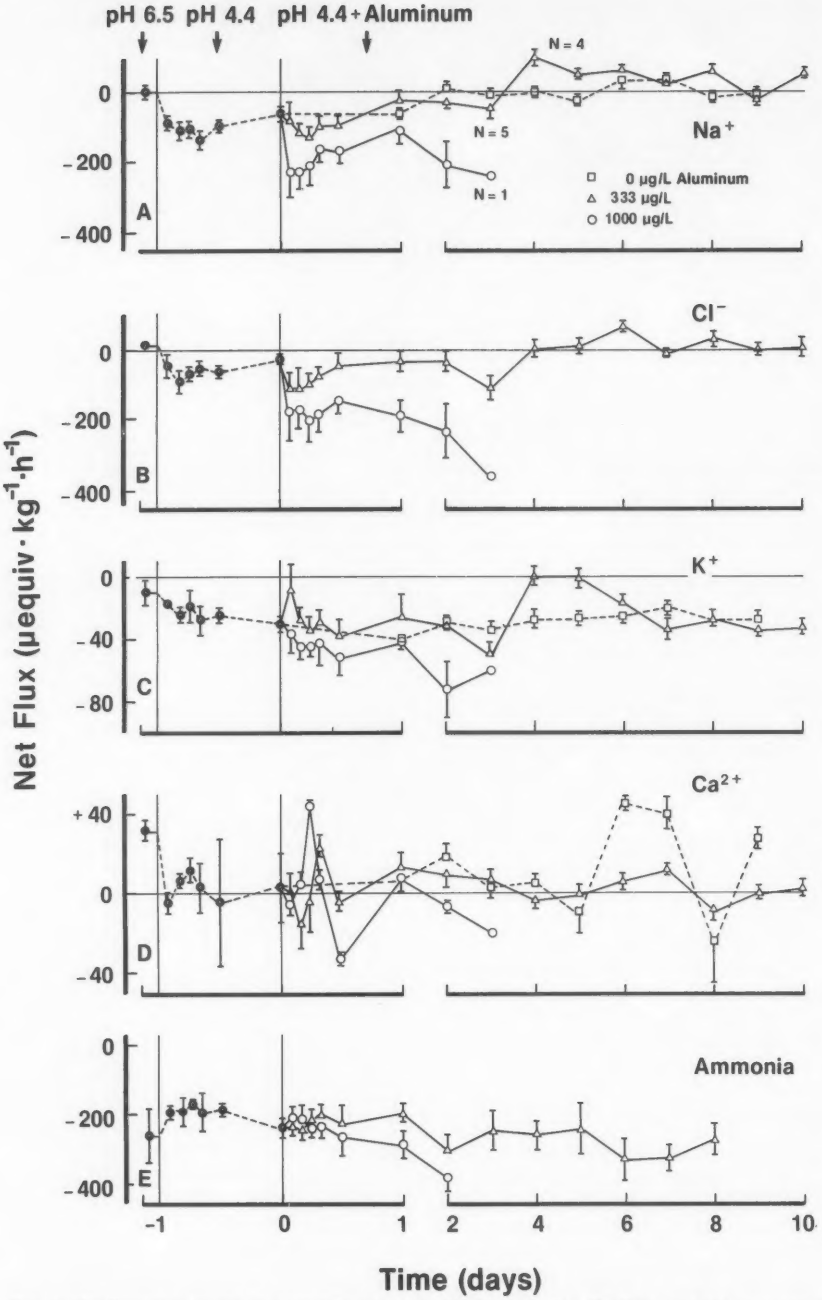


FIG. 2. Net flux rates (means ± 1 SEM) for (A) $Na^+$, (B) $Cl^-$, (C) $K^+$, (D) $Ca^{2+}$, and (E) ammonia at pH 4.4 and 0 and 333 and 1000 µg Al/L. Water $Ca^{2+}$ concentration = 25 µequiv/L. $N = 6$ for each treatment except where noted in Fig. 2A; reduction in $N$ is due to Al-induced mortality. Ion flux rates of initial 24 h period (pH 4.4) are pooled means for the three treatment groups (i.e. $N = 18$). Al exposure started on day 1. Note expanded scales for the net fluxes of $K^+$ and $Ca^{2+}$ and note change in time scale at day 1.

water. Tissue Al concentrations were determined by standard additions of known quantities of Al to the unknown samples. Levels of $^{22}Na^+$ in 5-mL water samples were measured either by gamma counting in a well-counter (Nuclear Chicago Model 1085) or by scintillation counting (LKB-Wallac). For scintillation counting the water samples were added to 10 mL of

TABLE 2. Summary of fish mortality at various combinations of pH, Al, and $Ca^{2+}$. Each cell started with six fish. Significant differences between high- and low-$Ca^{2+}$ cells were assessed using either a chi-square test (all high- and low-$Ca^{2+}$ cells combined) or Fisher's exact probability test (individual cell comparisons). Asterisks indicate significant effects of $Ca^{2+}$ ($p < 0.05$).

| pH | Al (μg/L) | Mortalities at $Ca^{2+}$ concentration (μequiv/L) 25 | 400 |
|---|---|---|---|
| 5.2 | 0 | 0 | 0 |
| | 111 | 0 | 0 |
| | 333 | 6* | 0 |
| 4.8 | 0 | 0 | 0 |
| | 111 | 0 | 0 |
| | 333 | 5* | 1 |
| 4.4 | 0 | 0 | 0 |
| | 333 | 2 | 0 |
| | 1000 | 6 | 6 |
| Combined | | 19* | 7 |

Aqueous Counting Scintillant (Amersham). Hematocrit was determined by centrifugation at 5000 × *g* for 5 min.

### Statistical Analysis

Data are reported throughout as means ± 1 SEM. Differences in mortalities between high- and low-$Ca^{2+}$ cells were evaluated using a 2 × 2 contingency table and either a chi-squared test (for $N > 20$) or Fisher's exact probability test for smaller sample sizes (Siegel 1956). Differences between groups were evaluated using the Student's unpaired *t*-test. Interrelationships between ion losses and water pH (at constant or zero Al) or ion losses and Al (at constant pH) were evaluated using least squares regression analysis.

## Results

### Al Toxicity

In low-$Ca^{2+}$ water (25 μequiv/L), there was increasing mortality with increasing Al and virtually 100% mortality at the highest Al concentration at each pH level (Table 2). The toxicity of 333 μg Al/L at low $Ca^{2+}$ decreased with a decrease in pH; mortality was 100% at pH 5.2 but only 33% at pH 4.4. Water $Ca^{2+}$ had a significant protective effect ($p < 0.05$, Table 2). In low-$Ca^{2+}$ waters, the various pH/Al combinations caused 35% mortality overall (19 fish out of 54). In contrast, the mortality in high-$Ca^{2+}$ water was only 13% (7 fish out of 54) for the same pH/Al combinations. All fish deaths occurred between 1 and 5 d after exposure to Al, but in low $Ca^{2+}$ the majority of the deaths (79%) occurred within 2 d whereas in high $Ca^{2+}$, only two out of the total of seven deaths had occurred by this time. There were no differences in either mortality or net ion fluxes in fish exposed to the same pH/Al/$Ca^{2+}$ combinations using $Ca(NO_3)_2$ and $AlK(SO_4)_2$ versus $CaCl_2$ and $AlCl_3$. Thus, results with the different salts have been combined.

### Net Ion Fluxes

Fish exposed to reduced water pH with and without Al suffered marked disturbances to internal ionic balance, as indicated by high rates of $Na^+$ and $Cl^-$ loss to the ambient water (Fig. 2–4). The patterns of ion loss were qualitatively similar for all 18 combinations of pH and Al. Therefore, rather than present each treatment individually, the results of three sets of experiments, that of pH 4.4, and 0, 333, and 1000 μg Al/L at low $Ca^{2+}$ (25 μequiv/L), are shown to illustrate typical responses (Fig. 2).

In fish at circumneutral pH, acclimated to the flux chambers for 24–48 h, the mean net flux rates for $Na^+$ and $Cl^-$ were not significantly different from zero ($p < 0.05$, paired *t*-test), indicating that the animals were in ion balance. Sudden exposure to pH 4.4 caused a substantial net efflux of $Na^+$ and $Cl^-$ within 2–3 h (Fig. 2A, 2B). Peak rates of $Na^+$ and $Cl^-$ loss occurred after 4–6 h at pH 4.4 and were of approximately equal magnitude. The rates of $Na^+$ and $Cl^-$ loss subsequently declined, although after 24 h at pH 4.4, they had not returned to zero.

In those fish maintained at pH 4.4 without Al exposure, net $Na^+$ and $Cl^-$ flux rates returned to zero approximately 48–72 h after the start of low pH exposure and remained at this level for the duration of the experiment (Fig. 2A, 2B; $Cl^-$ flux rates not shown). No mortality occurred in the six fish kept in these conditions.

Upon exposure to 333 μg Al/L after 24 h at pH 4.4, fish showed increased rates of $Na^+$ and $Cl^-$ loss for several hours followed by gradually decreasing ion losses over the next 48–72 h (Fig. 2A, 2B). However, two of the six fish died during Al exposure, one within 72 h and another within 120 h. The survivors appeared to recover some of their initial $Na^+$ and $Cl^-$ losses, as indicated by periods of net $Na^+$ and, to a lesser extent, net $Cl^-$ uptake. For the remaining 3–4 d of the experiment, net $Na^+$ and $Cl^-$ flux rates were not significantly different from zero.

When fish were exposed to 1000 μg Al/L after 24 h at pH 4.4, $Na^+$ and $Cl^-$ efflux rates increased rapidly to peak levels approximately twofold higher than losses from fish at 333 μg Al/L (Fig. 2A, 2B). Over the next 48 h, the fish all continued to lose $Na^+$ and $Cl^-$ at relatively high rates and all died within 72 h of the initial Al exposure.

Exposure to low pH and Al also caused brook trout to lose $K^+$ in a pattern similar to that for $Na^+$, with the highest rate of $K^+$ efflux occurring at the highest Al concentration (Fig. 2C). Although the patterns of $Na^+$ and $K^+$ loss were generally similar, the rate of $K^+$ loss was typically only about 20% of that for $Na^+$. Furthermore, $K^+$ balance remained negative throughout the 11-d exposure at pH = 4.4 and, Al = 0 μg/L. In contrast, low pH and Al exposure had no detectable effect on net $Ca^{2+}$ flux rates, which fluctuated on either side of zero, even in dying fish (Fig. 2D).

There was little change in the rate of ammonia excretion in response to pH 4.4. However, after 1 – 2 d of exposure to 333 and 1000 μg Al/L, ammonia excretion rates had increased by 30–50% (Fig. 2E). This phenomenon is likely stress related, resulting from a cortisol-mediated increase in protein catabolism (cf. McDonald 1983a).

From the above results it is evident that net $Na^+$ and $Cl^-$ flux rates were very sensitive indicators of low pH and Al stress. Since water $Cl^-$ levels in these experiments were near the lower limit of detection by coulometric titration, measurements of $Cl^-$ flux rates were subject to greater error than were $Na^+$ flux rates. Therefore, further analysis of ionic disturbances will largely concentrate on $Na^+$ fluxes.

Since the "instantaneous" $Na^+$ flux rates changed with time over the course of the experiments, they did not indicate the

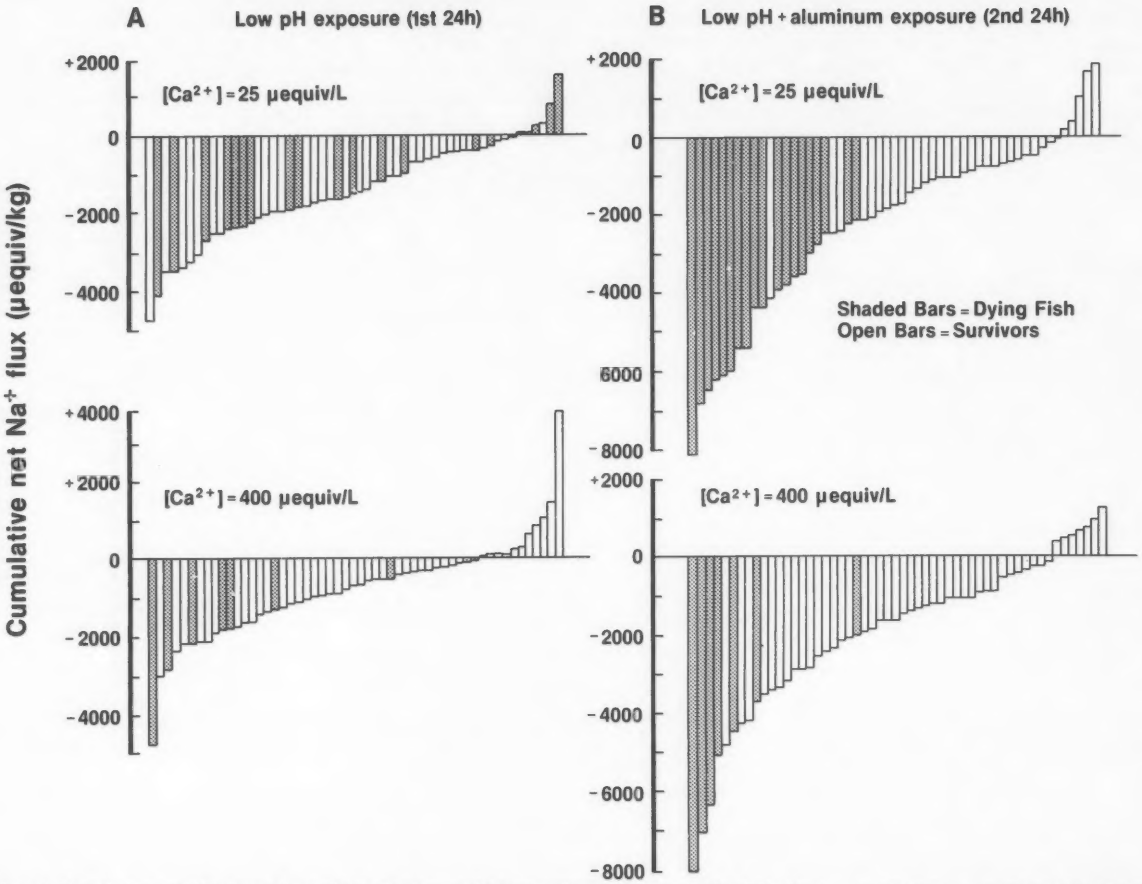


FIG. 3. Cumulative net $Na^+$ fluxes for individual fishes, ranked according to the magnitude of the flux: − = net loss; + = net uptake. Open bars indicate fish that survived for the duration of the experiment (i.e. 11 d) and shaded bars indicate fish that died subsequent to the first 24 h of Al exposure. (A) $Na^+$ fluxes over the first 24-h period of the experiment in which fish were exposed to low pH alone (pH 4.4, 4.8, or 5.2). (B) $Na^+$ fluxes over the second 24-h period in which fish were exposed to Al (0, 111, 333, or 1000 µg/L) plus low pH.

magnitude of the $Na^+$ losses incurred by the fish. The net $Na^+$ flux rates were therefore integrated over time to give cumulative net $Na^+$ fluxes. Cumulative net $Na^+$ fluxes were calculated individually for each fish for the first and second 24-h periods of the experiment, before any mortality occurred. This allows a comparison of $Na^+$ losses between fish which later died during the experiments (shaded bars, Fig. 3) and fish which survived (open bars).

There was considerable variation amongst fish in net $Na^+$ losses either when the fish were exposed to low pH (4.4, 4.8, or 5.2) over the first 24 h (Fig. 3A) or over the second 24 h (Fig. 3B), when exposed to low pH (4.4, 4.8, or 5.2) plus Al at various concentrations (0, 111, 333, or 1000 µg/L). Here, the protective effect of $Ca^{2+}$ can clearly be seen in the generally smaller sodium losses at 400 versus 25 $Ca^{2+}$ and the disruptive effect of Al by the greater $Na^+$ losses (Fig. 3A versus 3B). During the initial 24 h, there was no apparent relationship between the amount of $Na^+$ loss and eventual mortality. However, when fish were subjected to a combination of low pH and elevated Al in the second 24 h, there were significantly greater $Na^+$ losses in those fish which subsequently died. The lethal threshold of ion loss in low $Ca^{2+}$ (for the second 24 h) was approximately 2000 µequiv $Na^+$/kg or approximately 4% of total body $Na^+$ (53 mequiv/kg, cf. Shearer 1984). Of the 19 fish that died in the low-$Ca^{2+}$ group, all lost in excess of this amount and only five of survivors lost more. In the high-$Ca^{2+}$ group, the apparent threshold was substantially higher, in excess of 3500 µequiv/kg. Six of the seven fish that died and only three of the 47 survivors in the high-$Ca^{2+}$ group exceeded this level of loss. The marked difference between survivors and nonsurvivors is perhaps best illustrated by an example from one cell (pH 4.4, 333 µg Al/L, 25 µeq $Ca^{2+}$/L) which had two mortalities out of six fish. In this cell the survivors (240-h exposure) lost 1100 ± 220 µequiv $Na^+$/kg over the first 24 h of Al exposure whereas the two nonsurvivors (which died after 72 and 96 h) lost 5010 and 2300 µeq/kg, respectively, during this period.

The mean cumulative net $Na^+$ fluxes for the second 24-h period (low pH plus Al) were used to assess the interactive effects of pH and Al on $Na^+$ balance (Fig. 4). Considering the low-$Ca^{2+}$ group first, in the absence of Al, fish showed a net uptake of $Na^+$ at pH 5.2 while sustaining net losses of $Na^+$ at the two lower pH levels (Fig. 4A). At each of the three pH levels tested, there was a significant direct correlation between

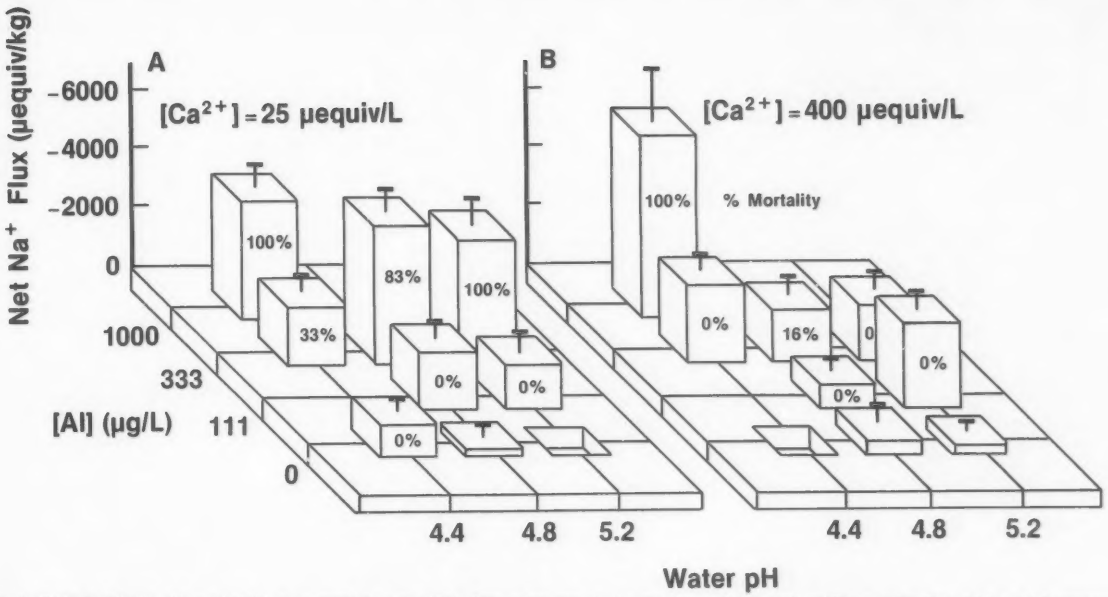


FIG. 4. Effects of pH, Al, and $Ca^{2+}$ on cumulative net $Na^+$ fluxes (means ± 1 SEM, $N = 6$ for each cell) over the first 24 h of Al exposure (follows 24 h at same pH without Al). Exposure conditions that were not employed are indicated by the absence of a bar (e.g. 111 µg Al/L at pH 4.4).

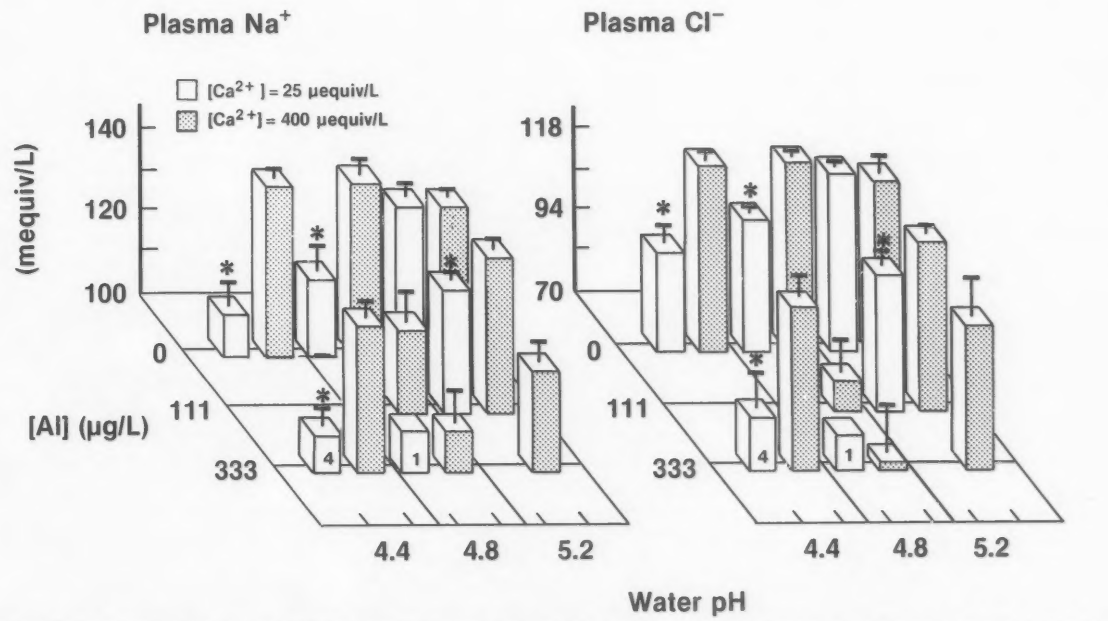


FIG. 5. Effects of water pH, Al, and $Ca^{2+}$ on plasma concentrations of $Na^+$ and $Cl^-$. Terminal samples, survivors only, $N = 6$ except where noted. Asterisks indicate significant differences ($p < 0.05$, unpaired $t$-test) between high- and low-$Ca^{2+}$ animals at the same pH/Al exposure.

Al concentration and net $Na^+$ efflux ($r = 0.670$, 0.851, and 0.789 for pH 4.4, 4.8, and 5.2, respectively), with the highest $Na^+$ losses associated with high mortality (Fig. 4). Also, at 333 µg Al/L, $Na^+$ losses were significantly higher at pH 4.8 than at pH 4.4 ($p < 0.01$, unpaired $t$-test).

In high-$Ca^{2+}$ water, the significantly lower mortality (Table 2) was associated with lower and more variable cumulative net $Na^+$ fluxes (Fig. 4B). Nonetheless, in all cases except pH 4.8 and 111 µg Al/L, Al caused significantly greater ion losses than low pH alone ($p < 0.01$, unpaired $t$-test).

## Plasma Ion Concentrations

Ion levels (Fig. 5) in terminally collected blood samples (taken from surviving fish at the end of the experiments)

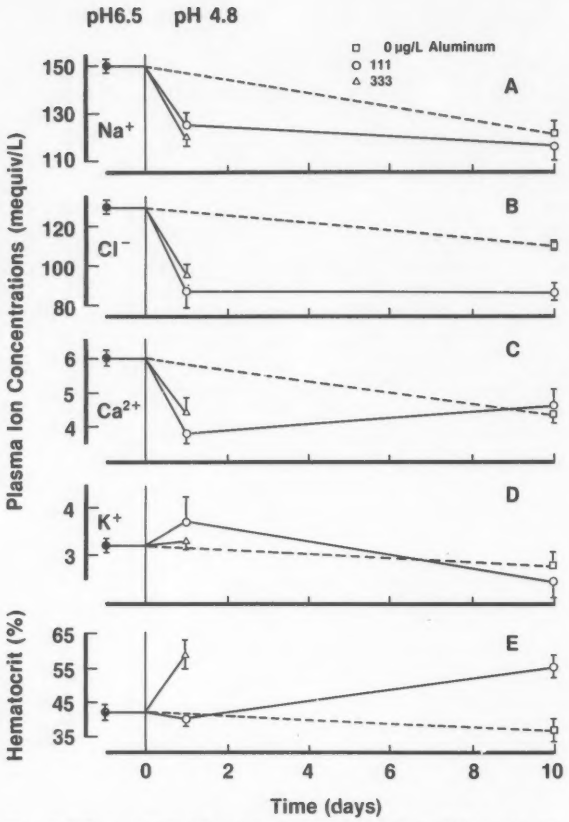


FIG. 6. Effects of pH 4.8 and 0 and 111 and 333 µg Al/L on (A–D) plasma ion concentrations (E) and hematocrit. Water $Ca^{2+}$ concentration was 25 µequiv/L. Terminal samples, survivors only, $N = 6$ in acid-exposed fish. For fish at pH 6.5, $N = 10$; blood samples were drawn by caudal puncture after 18 d of acclimation to artificial salt water ($Ca^{2+} = 25$ µequiv/L).

reflected, to a large extent, the effects of net $Na^+$ and $Cl^-$ losses to the medium. In all groups of fish, plasma $Na^+$ and $Cl^-$ levels were reduced relative to control fish maintained at pH 6.5 and acclimated to soft water. In control fish, plasma $[Na^+]$ and $[Cl^-]$ were 149 ± 1 and 133 ± 1 mequiv/L, respectively ($N = 10$; data plotted on Fig. 6).

The blood ion data confirm the major protective effect of $Ca^{2+}$; in virtually every cell, plasma $Na^+$ and $Cl^-$ levels were significantly higher in the high-$Ca^{2+}$ survivors (shaded bars, Fig. 5) than in the low-$Ca^{2+}$ survivors (open bars) for the same pH/Al exposure. These data also reveal some important effects of sublethal exposures not readily apparent from initial $Na^+$ losses (Fig. 4). First, in those low-$Ca^{2+}$ animals not exposed to Al, plasma ion levels correlated well with pH ($r = 0.847$), with the lowest levels at pH 4.4. In contrast, no effect of low pH (without Al) was seen in the high-$Ca^{2+}$ animals. Second, there was a clear effect of Al exposure on high-$Ca^{2+}$ animals; at each pH (pH 4.4, 4.8, 5.2) there was a significant inverse correlation ($r \geq 0.545$) between Al concentration and plasma NaCl concentration. In contrast, no plasma ion/Al correlation was evident in low-$Ca^{2+}$ animals despite the correlation between Al concentration and net ion losses in this group (Fig. 4). However, it should be noted that the plasma data are terminal samples for survivors only. Thus, animals that lost a large amount of NaCl at the higher Al concentrations (Fig. 4) had already died when the terminal samples were collected.

Figure 6 gives an indication of the time course of the plasma ion disturbances. These data are for fish exposed to pH 4.8 and Al concentrations of 0, 111, and 333 µg/L, and in this case, blood samples were collected in two experimental series, one terminated after 1 d of Al exposure and the other after 10 d of Al exposure. These data show that most of the plasma NaCl depressions resulting from pH and Al exposure occurred in the first 24 h (Fig. 6A, 6B). Little difference was seen between 111 and 333 µg/L in terms of plasma NaCl loss, although the increase in hematocrit (Fig. 6E), which is typically secondary to ion loss (cf. Milligan and Wood 1982), was much larger at 333 µg/L than at 111 µg/L. Furthermore, five out of the six fish at 333 µg/L died whereas the six fish at 111 µg/L survived. Fish exposed to 111 µg Al/L showed a significant decrease in plasma $Ca^{2+}$ concentration within 24 h (Fig. 6C). However, at the end of the 11-d experiment, $Ca^{2+}$ concentration was not significantly different from control values in all fish (unpaired *t*-test). Little variation was seen in plasma $K^+$ (Fig. 6D) despite persistent net $K^+$ losses from the animals (Fig. 2C). This is likely due to the substantial intracellular reserve of $K^+$.

## Unidirectional $Na^+$ Fluxes

At periodic intervals, the whole-animal net $Na^+$ fluxes were partitioned into their influx and efflux components by means of $Na^+$ radiotracer disappearance from the medium (Fig. 7). Since these measurements were for 30 min only, they were, in effect, "snapshots" and did not have the resolution of the cumulative $Na^+$ losses (Fig. 3, 4) or the terminal plasma measurements (Fig. 5, 6). For example, based on these measurements, there was virtually no effect of water $Ca^{2+}$ concentration on either influx or efflux (although the net flux data and plasma ions indicate that such effects should exist). Nor were there any consistent differences between those animals that were exposed to low pH only and those exposed to sublethal levels of Al (111 µg/L at pH 5.2, 333µg/L at pH 4.4). Nevertheless, the results (Fig. 7) do provide some insights into the specific disturbances caused by $H^+$ and Al. To simplify the presentation of these results, the data for high and low $Ca^{2+}$ and zero and sublethal Al have been pooled at each pH.

The initial effect of low pH and/or Al exposure was invariably a combination of influx inhibition and a marked efflux stimulation (Fig. 7A, 7B). While the pH (alone) effect was mainly on influx (Fig. 7A), the effect of Al at low pH was on both influx and efflux (Fig. 7B), inhibiting the former and increasing the latter. The efflux effect was larger and therefore probably the main reason for the initial ion losses (Fig. 3), but it was time dependent, being most pronounced in any one treatment over the first 2–8 h of exposure and then subsequently declining. Efflux typically returned to near control levels within 24 h (Fig. 7C) in all but the acutely lethal exposures. In contrast, the effects of low pH and sublethal Al exposure on $Na^+$ influx were quite persistent. In all fish at low pH alone, influx remained significantly inhibited relative to controls at pH 6.5, with means varying between 20 and 60% of initial values (Fig. 7C). Since these fish were, for the most part, nearly in $Na^+$ balance (e.g. Fig. 2) from day 3 onward, their $Na^+$ effluxes must have been consistently less than initial values as well.

The acute effects of Al on $Na^+$ fluxes were similar to but greater than those of low pH alone. The main initial effects were

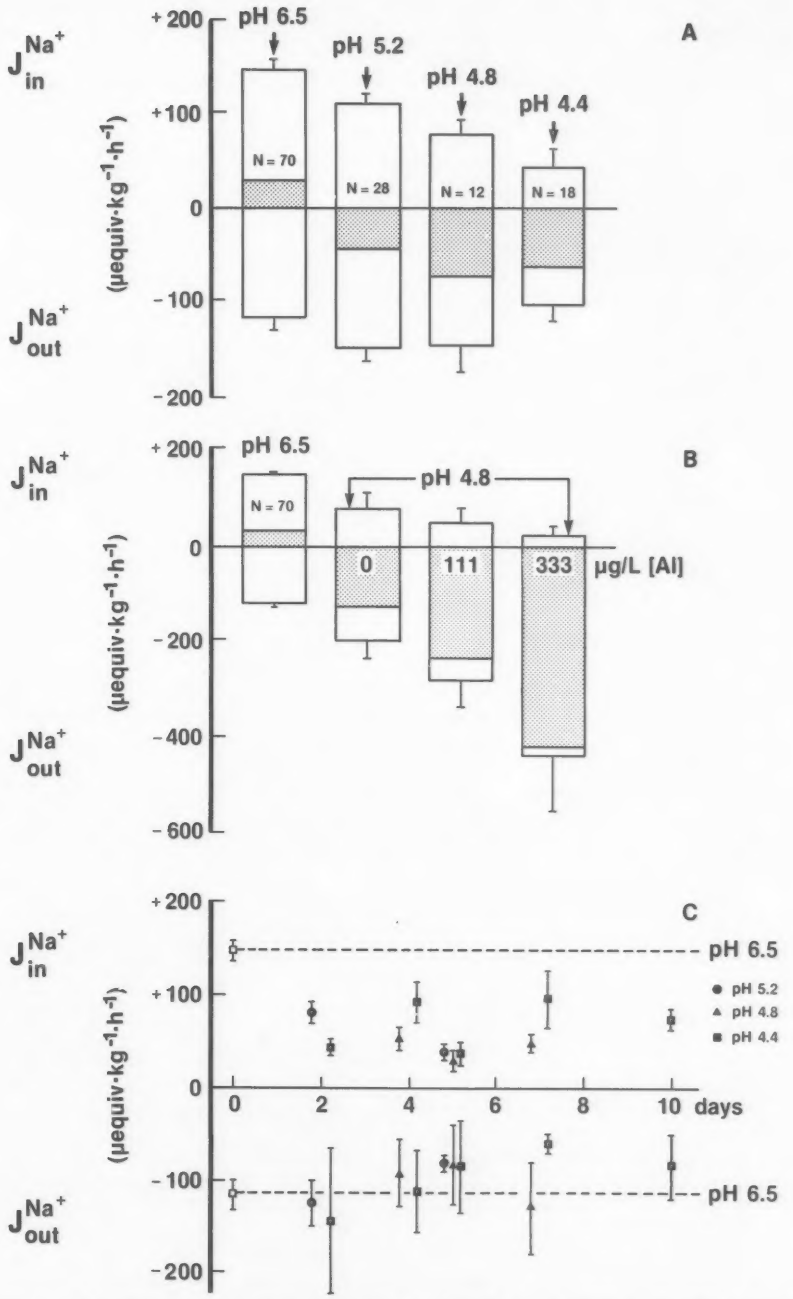


FIG. 7. Unidirectional $Na^+$ fluxes ($J_{in}$ and $J_{out}$) as determined by radiotracer disappearance from the water over 30 min. Net flux ($J_{net}$) is indicated by the shaded areas. Data from experiments on low-$Ca^{2+}$-acclimated animals have been pooled with those from high-$Ca^{2+}$-acclimated animals. (A) Acute effect of low pH. For pH 5.2, 4.8, and 4.4, measurements were made after 8 h of low pH exposure. Means ± 1 SEM, $N$ as indicated. (B) Acute effect of Al exposure. Measurements were made after 6 h of exposure to Al. Means ± 1 SEM, $N = 6$ except where noted. Note different scale from Fig. 7A. (C) Effect of duration of low pH exposure. Measurements made at pH 5.2 have been pooled with those made at pH 5.2 and 111 μg Al/L; measurements made at 4.4 have been pooled with those made at pH 4.4 and 333 μg Al/L. Means ± 1 SEM, $N$ = 6–24.

a concentration-dependent stimulation of efflux above that caused by low pH alone and a slight further inhibition of $Na^+$ influx (Fig. 7B). With time, these effects abated in survivors to the point that the influxes and effluxes in sublethally Al-exposed fish (111 μg/L at pH 5.2, 333 μg/L at pH 4.4) were indistinguishable from fish exposed only to low pH (Fig. 2).

### Tissue Al Levels

All Al-exposed fish accumulated Al in their gills, but this was apparently mainly surface or subsurface bound, since no internal Al (plasma or liver) could be detected. In surviving fish, gill Al concentrations were relatively independent of water pH and $Ca^{2+}$ but were dependent on water Al, averaging 235 ± 47 μg/g wet tissue ($N$ = 24) at 111 μg/L and 413 ± 75 μg/g ($N$ = 22) at 333 μg/L. In nonsurviving fish, gill Al levels were significantly higher ($p < 0.01$, unpaired $t$-test) than in survivors, averaging, for all Al cells combined, 837 ± 98 μg/g ($N$ = 25).

## Discussion

### Toxic Action of Al

The results of the present study confirm previous findings (Muniz and Leivestad 1980; Neville 1985) in showing that Al exposure produces disturbances to ion balance that resemble those caused by exposure to low pH alone (reviewed by Wood 1987). Our study now clearly shows that transepithelial ionic fluxes and plasma ion concentrations become more severely disrupted with increasing concentration of either $H^+$ or Al and that the disturbance is primarily to NaCl balance and not to other body electrolytes. However, the major contribution of the present study is to offer new insights into the specific nature of the toxic action of Al upon gill ionoregulatory mechanisms and how that toxic action is modified by duration of exposure and by the pH and $Ca^{2+}$ concentration of the external environment.

The results show that the ionoregulatory response of fish to Al/low pH exposure can be divided into two relatively distinct phases: an initial "shock" phase and a "recovery" phase. The shock phase is characterized by two responses: a pronounced reduction of ionic uptake which likely reflects an inhibition of the branchial transport ATPase's (Staurnes et al. 1984) and a large stimulation of passive ionic efflux which likely arises from an increase in the paracellular ionic permeability of the gill epithelium (McDonald 1983b; Marshall 1985). In this initial phase, passive ion efflux is the main contributor to net ion losses and in some animals can be of sufficient magnitude to cause death. The recovery phase, in contrast, is seen only in survivors and is incomplete, at least under the pH/Al conditions employed in this study. It is characterized by little, if any, recovery of uptake and by a reduction in efflux to control levels or less. In this phase, net ion losses arise largely from the persistent inhibition of uptake. In an earlier study (McDonald et al. 1983), we showed that rainbow trout (*Salmo gairdneri*) respond to acid stress in this fashion and also showed that the behavior of $Cl^-$ fluxes was essentially the same as that of $Na^+$. In the present study, the failure of $Na^+$ influx (and by extension, $Cl^-$ influx) to fully recover appears to be the main persistent effect of sublethal low pH and Al exposure. To defend ion content, or to recover initial ion losses, these fish must rely on a reduction of efflux across the gills. This, in turn, appears to be the major adaptive response to Al/low pH exposure (see below).

The initial shock response is apparently critical to survival, for the magnitude of the initial $Na^+$ loss is closely related to whether or not a fish survives a particular Al/low pH exposure (Fig. 3). The difference in ion loss between survivors and nonsurvivors, best illustrated by Fig. 3, is striking, particularly since a homogeneous population of brook trout was used (all from the same hatchery, all apparently in the same physiological state prior to use). A plausible explanation for this difference is that there is a critical threshold of initial disturbance to gill function. If this threshold is exceeded the result is the establishment of a positive feedback relationship between Al-induced epithelial damage and ion loss. In this circumstance, one can envision $Al/H^+$ interactions setting off a host of physiological and morphological reactions: displacing membrane-bound $Ca^{2+}$, weakening tight junctions, and deforming and lifting the lamellar epithelium (Chevalier et al. 1985), causing increased exposure of binding sites in the gills and further gill Al accumulation and acceleration of ion loss leading to death. This type of interaction would explain the observation of higher gill Al content in nonsurvivors compared with survivors. By the same reasoning, the initial damage in surviving fish would be below the critical threshold, allowing time for compensatory mechanisms to operate. The most useful function of these mechanisms would be the reduction of epithelial ion leakiness and/or protection of the epithelium from further surface effects of Al. The present results suggest that ion leakiness does seem to decline, based on the measurements of $J_{out}$ (Fig. 7C versus 7A and 7B), but do not reveal how such changes could come about. A small contributing factor will be the reduction of the plasma to water NaCl diffusion gradient by the initial losses. However, the overall adaptive response may, in fact, be the result of a whole suite of adjustments: increased mucous secretion, cell volume changes that reduce paracellular channel permeability, the formation of stable and relatively innocuous polymeric Al precipitates, sequestering of Al in the intracellular compartment, repairing damaged cells, hormonal adjustments to epithelial permeability, and even changes in gill perfusion. Although there is an extensive literature on the morphological character of gill lesions induced by Al (Chevalier et al. 1985; Karlsson-Norrgren et al. 1986a, 1986b) and other toxicants (reviewed by Mallatt 1985), there is very little information on the morphological or physiological basis for the damage repair or adaptive mechanisms of the gills. This is clearly a very fruitful area for further research.

### Interaction of Al and $H^+$

Although the toxicites of Al and $H^+$ resemble one another, they are not strictly additive, since, as demonstrated here and in previous toxicity studies, increasing acidity (at least over the pH range of 5–4) actually reduces the toxicity of Al. For example, Schofield and Trojnar (1980) showed that the median survival time ($LT_{50}$) of brook trout fry exposed to 1000 μg Al/L increased from 1.8 d at pH 4.9 to 5 d at pH 4.0. In the present study the relationship was similar but less straightforward; Al (at least at low $Ca^{2+}$) was about three times as toxic at pH 4.8 as at pH 4.4 (based on similar ion losses at 333/pH 4.8 and at 1000/pH 4.4, Fig. 5) but was either of similar or lower toxicity at pH 5.2 depending on water $Ca^{2+}$ (similar initial ion losses at 5.2/333 and at 4.8/333 in low $Ca^{2+}$, Fig. 4; lower terminal plasma NaCl at 4.3/333 than at 5.2/333 in high $Ca^{2+}$, Fig. 5).

Underlying this complex interaction of Al and pH are two important aspects of Al chemistry in water; Al solubility drops

by a factor of 72 (Fig. 1B) over the pH range of 4.4–5.2 and is accompanied by major changes in speciation (Fig. 1A), from mostly trivalent Al at pH 4.4 to mostly monovalent and divalent hydroxides at pH 5.2. Another important factor in this context is the pH of the microenvironment at the gill surface. Although the gill surface is extremely difficult to characterize chemically, it is most likely alkaline relative to the immediately adjacent interlamellar water. Factors contributing to this condition are buffering by the polyanionic mucus layer anchored to the surface (Kirschner 1978) and the excretion of ammonia in the basic form, $NH_3$ (Wright and Wood 1985). Furthermore, the subsurface environment (extracellular fluids of the gill epithelium, and by extension the intracellular fluids) will contribute to this alkalinity, since it is likely to remain close to neutrality throughout Al exposure under even the most acutely lethal conditions (see Wood et al. 1988).

With this background in mind, two distinct possibilities can be suggested for a mechanism of Al toxicity, one based on solubility and the other based on speciation.

The first (as suggested by Schofield and Trojnar 1980) views Al toxicity as a function of the transformation of soluble monomeric Al species into either polymers and/or precipitates at the surface, this transformation being principally responsible for the gill damage that causes physiological disturbances. Increasing the water pH would enhance this process, providing, of course, that the pH of the surface microenvironment follows that of the surrounding water.

The second possible mechanism is based on differences in toxicity amongst the Al species. Here, the suggestion is that Al species vary in terms of their reactivity with surface binding sites and that polymerization and/or precipitation are secondary processes. This hypothesis would explain how Al could be more toxic at 4.8 than at 5.2, providing one assumes that the polycationic Al species ($Al^{3+}$, $Al(OH)^{2+}$) are more toxic than the monovalent hydroxide ($Al(OH)_2^+$). At pH 4.8 the former make up 84% of the total soluble Al, but this declines to 58% at pH 5.2 (Fig. 1A). The lower toxicity of pH 4.4 versus 4.8 could then, in turn, be explained if the divalent cation is more toxic than the trivalent cation.

However, it is important to emphasize that the two toxic mechanisms are probably not mutually exclusive. Under some circumstances, both may be equally active in producing gill damage whereas under others, one may predominate over the other. Factors determining the predominance of one over the other could include the pH or $Ca^{2+}$ concentration of the external environment or simply the duration of exposure if there are major adaptive changes occurring at the surfaces of the gills. The observation by Wood et al. (1988) that respiratory distress becomes greater and ionic disturbance less when $Ca^{2+}$ is elevated at constant pH and Al (pH 4.8/333 μg/L) lends support to the notion that Al can operate by more than one toxic mechanism and that its action is very much dependent on exposure conditions.

### Protective Effect of $Ca^{2+}$

The range of water $Ca^{2+}$ employed in the present study (0.025–0.4 mequiv/L) may not seem large but, in fact, it represents the full range found in waters sensitive to acidification (i.e. those with low calcium bicarbonate concentrations and therefore low acid neutralizing capacity, Harvey et al. 1981). Furthermore, this range is sufficient to provide a major protective effect. Based on present results, $Ca^{2+}$ protects largely by reducing ion loss, thereby reducing mortality (Table 2; Fig. 3). Furthermore, $Ca^{2+}$ is similarly antagonistic to low pH alone (Fig. 3, 6) and to Al, in combination with low pH (Fig. 3, 5).

The protective effect of $Ca^{2+}$ is now well established in the toxicology literature as reducing the toxicity not only of $H^+$ and Al but of a number of other aquatic contaminants as well (e.g. Brown 1968; Alabaster and Lloyd 1980). The protection is thought to arise from weak ionic interactions between $Ca^{2+}$ and surface ligands (membrane integral and peripheral proteins, mucopolysaccharides, anionic residues in intercellular cements). These interactions act to stabilize apical membranes and increase the tightness of intercellular tight junctions (reviewed by McDonald 1983b), thereby reducing permeability of the membrane and increasing its resistance to attack by surface-active toxicants. Therefore, much of the effect of Al and $H^+$ may be related to competitive interactions with $Ca^{2+}$ for gill anionic sites. Furthermore, the result of increasing water $Ca^{2+}$ may be to delay or reduce the binding of Al at these sites. Instead, high water $Ca^{2+}$ may promote polymerization or precipitation of Al on the surface, since neither process would require prior binding of Al to gill anions. The end result may not necessarily be a change in the gill Al content so much as a difference in the form and reactivity of the Al present. Thus, one could explain the observation of greater respiratory and lower ion disturbance at high $Ca^{2+}$ (Wood et al. 1988) if the result of this process was the formation of a thicker surface coat with a greater diffusion distance. The precise nature of Al and $Ca^{2+}$ interactions at the gill surface is presently under investigation in our laboratories.

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# Blood Gases, Acid–Base Status, Ions, and Hematology in Adult Brook Trout (*Salvelinus fontinalis*) under Acid/Aluminum Exposure

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Wood, C. M., R. C. Playle, B. P. Simons, G. G. Goss, and D. G. McDonald. 1988. Blood gases, acid–base status, ions, and hematology in adult brook trout (*Salvelinus fontinalis*) under acid/aluminum exposure. Can. J. Fish. Aquat. Sci. 45: 1575–1586.

The relative importance of ionoregulatory and respiratory disturbances in brook trout (*Salvelinus fontinalis*) under acid/Al stress in soft water is dependent upon water pH and $Ca^{2+}$ levels. Trout acclimated to $Ca^{2+}$ = 25 or 400 μequiv/L were fitted with arterial catheters and exposed to acid/Al for 10 d under flow-through conditions. Parameters monitored included pHa, $Pa_{O_2}$, $Pa_{CO_2}$, $HCO_3^-$, $\Delta H^+m$, $Na^+$, $Cl^-$, $K^+$, $Ca^{2+}$, protein, lactate, glucose, hemoglobin, and hematocrit. Exposure to pH = 4.8 (no Al) at $Ca^{2+}$ = 25 μequiv/L caused no mortality and negligible physiological disturbance. Addition of Al (333 μg/L or 12.3 μmol/L) resulted in >80% mortality (LT50 = 39.0 h) preceded by a marked decrease of plasma $Na^+$ and $Cl^-$, a moderate disturbance of blood gases, but no acidosis. At higher $Ca^{2+}$ (400 μequiv/L), this same exposure (pH = 4.8, Al = 333 μg/L) caused similar mortality (LT50 = 38.5 h) but smaller ionic disturbances, much larger decreases in blood $O_2$, increases in blood $CO_2$, and respiratory acidosis. Exposure to pH = 4.4 (no Al) at $Ca^{2+}$ = 25 μequiv/L caused 60% mortality (LT50 = 170.0) preceded by marked ionic disturbances and metabolic acidosis, but little change in blood gases. Addition of Al (333 μg/L) increased mortality to >80% (LT50 = 78.2 h) with smaller ionic but greater respiratory disturbances.

L'importance relative de perturbations ionorégulatoires et respiratoires chez l'omble de fontaine (*Salvelinus fontinalis*) soumis à un stress acide/Al en eau douce dépend du pH et des teneurs en $Ca^{+2}$ de l'eau. Des ombles acclimatés à des teneurs en $Ca^{+2}$ de 25 ou 400 μequiv/L ont été munis de cathéters artériels puis exposés pendant 10 d à diverses conditions acides/Al dans un milieu à débit continu. Les paramètres suivants ont été quantifiés : pHa, $Pa_{O_2}$, $Pa_{CO_2}$, $HCO_3^-$, $\Delta H^+m$, $Na^+$, $Cl^-$, $K^+$, $Ca^{+2}$, protéine, lactate, glucose, hémoglobine et hématocrite. L'exposition au pH 4,8 (absence d'Al) et à une teneur en $Ca^{+2}$ de 25 μequiv/L a entraîné une faible perturbation physiologique mais aucune mortalité. L'apport d'Al (333 μg/L ou 12,3 μmol/L) a entraîné un taux de mortalité supérieur à 80 % (TL50 = 39,0 h); la mort a été précédée d'une baisse marquée des teneurs en $Na^+$ et $Cl^-$ du plasma, d'une perturbation modérée de l'équilibre des gaz sanguins mais d'aucune acidose. À une teneur plus élevée en $Ca^{+2}$ (400 μequiv/L), cette même exposition (pH = 4,8, Al = 333 μg/L) a entraîné un taux de mortalité semblable (TL50 = 38,5 h) mais de plus faibles perturbations des ions, de plus grandes diminutions de la teneur en $O_2$ du sang, des augmentations de la teneur en $CO_2$ du sang et une acidose respiratoire. L'exposition au pH 4,4 (absence d'Al) à une teneur en $Ca^{+2}$ de 25 μequiv/L a donné un taux de mortalité de 60 % (TL50 = 170,0 h) précédée de perturbations de l'équilibre ionique et d'une acidose métabolique marquées mais de faibles variations de l'équilibre des gaz sanguins. L'apport d'Al (333 μg/L) a porté le taux de mortalité à plus de 80 % (TL50 = 78,2 h) et a été accompagné de plus faibles perturbations de l'équilibre ionique mais de plus grandes perturbations respiratoires.

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It is now clear that low environmental pH, ambient calcium level ($Ca^{2+}$), and aluminum concentration (Al) interact with one another in an extremely complex fashion in causing toxicity to fish (Schofield and Trojnar 1980; Muniz and Leivestad 1980a, 1980b; Baker and Schofield 1982; Wood and McDonald 1982; Howells et al. 1983; Brown 1983; Howells 1984; Neville 1985; McDonald et al. 1988; Wood 1988). In broad overview, the major trends appear to be as follows: (i) increases in toxicity are associated with decreasing pH, with increasing Al, and with decreasing $Ca^{2+}$; (ii) the effect of Al varies with pH, changing from a greatly exacerbating influence under moderately acidic conditions to a neutral or even protective one at very low pH; (iii) there is a general protective effect of $Ca^{2+}$ against both Al and acid toxicity, but the degree of protection is dependent upon the levels of these two toxicants; (iv) the toxic mechanism of pure acid stress involves only ionoregulatory disturbance, at least under environmentally realistic conditions of pH and $Ca^{2+}$; (v) the toxic mechanism of Al involves either ionoregulatory or respiratory disturbance or both, the severity and relative contribution of each being critically dependent upon the pH and absolute Al level.

This final conclusion is based on only a limited amount of physiological work involving measurements of ionic flux rates (Witters 1986; Booth et al. 1988), respiratory parameters (Rosseland 1980; Neville 1985), and blood gases, ions, and other circulating substances in single terminal blood samples (Muniz and Leivestad 1980a, 1980b; Leivestad et al. 1980; Neville 1985; Rosseland et al. 1986; Witters 1986; Booth et al. 1988). As yet there is no information on progressive changes in blood parameters in chronically catheterized animals during

acid/Al exposure. In the preceding paper (Booth et al. 1988), we have described the ion flux responses of noncannulated brook trout (*Salvelinus fontinalis*) to 10 d of exposure to a defined pH/$Ca^{2+}$/Al matrix in flowing soft water. In the present study, we report progressive changes in blood gases, acid–base status, electrolytes, metabolites, and hematology of chronically cannulated brook trout at selected levels of pH, $Ca^{2+}$, and Al. These levels were chosen to reflect those monitored during episodic acid surge events in the field while ensuring that the Al level (333 μg/L) was high enough to exert definite toxic effects but low enough to remain below the solubility limit at the tested pH's (Muniz and Leivestad 1980a; Schofield and Trojnar 1980; Johnson et al. 1981; McDonald et al. 1988). A pH of 4.4 is about the most severe acidity seen in the field, while 4.8 is more commonly encountered, but of little toxicity to brook trout by itself. However, these two pH's are far enough apart to greatly alter Al speciation. The $Ca^{2+}$ levels chosen (25 and 400 μequiv/L) bracket the range of natural soft water.

The objectives were to correlate internal changes in blood composition with the external flux rate effects described by Booth et al. (1988) and to understand the toxic mechanism(s) of acid and Al action in relation to water pH and $Ca^{2+}$ levels. Specifically we tested whether either lowered pH or elevated $Ca^{2+}$ protected against the toxic effects of Al and whether the balance between respiratory and ionoregulatory effects could be shifted by changes in pH and $Ca^{2+}$.

## Materials and Methods

### Experimental Animals

Experiments were performed on 81 adult brook trout (150–350 g) at McMaster University, Hamilton, Ontario (altitude = 100 m). The fish were obtained, held, and acclimated to artificial soft water ($Ca^{2+}$ = 25 or 400 μequiv/L, i.e. 0.5 or 8 mg/L; Na = 50–100 μequiv/L, i.e. 1.1–2.3 mg/L; pH = 6.3–6.7) as described by Booth et al. (1988). Feeding was stopped 7 d before cannulation to minimize possible effects on measured parameters of the unavoidable starvation during the experiments. All fish were fitted with chronic indwelling arterial catheters while under MS-222 anaesthesia (Sigma; 50 mg/L, buffered to pH ≃ 6.5). In preliminary trials, we found that dorsal aortic cannulation through the buccal cavity, as traditionally used for rainbow trout (*Salmo gairdneri*) (Soivio et al. 1972), had a lower success rate in brook trout because of slightly different anatomy. Instead, the caudal artery was exposed by a small incision in the peduncle, and a catheter (Clay–Adams PE50) was inserted into the dorsal aorta to about the level of the coeliac branch. The wound was dusted with the antibiotic Prefuran (Nifurpirinol, Argent Laboratories) and closed with silk suture. The catheter was filled with heparinized Cortland saline (Wolf 1963; Sigma sodium heparin, 50 IU/mL). After cannulation, fish were transferred to darkened Plexiglas chambers (volume = 2.7 L) of the design of McDonald and Rogano (1986) as used in the preceding study (Booth et al. 1988). Forty-eight to 72 h in flowing acclimation water was allowed for recovery before withdrawal of control blood samples.

### Test Conditions

Fish were acclimated for at least 2 wk to the $Ca^{2+}$ level used in the test condition. Experiments were performed at five different combinations of pH, $Ca^{2+}$, and Al, each involving 10 d of exposure to the test condition after sampling under the acclimation condition (control samples). The five combinations were chosen to assess Al effects at two different levels of acidity, to separate the effects of acid alone from acid plus Al stress, and to examine the influence of elevated ambient $Ca^{2+}$ on the responses to one acid plus Al treatment. The test conditions were as follows: (1) pH = 4.8, $Ca^{2+}$ = 25 μequiv/L, Al = 0 μg/L ($N$ = 10); (2) pH = 4.8, $Ca^{2+}$ = 25 μequiv/L, Al = 333 μg/L, i.e. 12.3 μmol/L ($N$ = 19); (3) pH = 4.8, $Ca^{2+}$ = 400 μequiv/L, Al = 333 μg/L ($N$ = 28); (4) pH = 4.4, $Ca^{2+}$ = 25 μequiv/L, Al = 0 μg/L ($N$ = 11); (5) pH = 4.4, $Ca^{2+}$ = 25 μequiv/L, Al = 333 μg/L ($N$ = 13). Test waters were prepared daily in 1500-L batches by addition of NaCl, $CaCl_2 \cdot 2H_2O$, $H_2SO_4$, and $AlCl_3 \cdot 6H_20$ (all analytical grade) to deionized water generated by reverse osmosis or deionizing resin cannisters. One important difference from the protocol of Booth et al. (1988) was that acid plus Al were delivered to the fish from time 0, rather than after an initial 24 h of acid exposure alone.

The $P_{O_2}$ of the inflowing water was maintained above 140 Torr and $P_{CO_2}$ less than 1 Torr. The pH of inflowing water was set slightly below the desired value because of the alkalinizing influence of the fish (largely due to ammonia excretion) on the water as it passed through the chamber. Flow to each chamber was set to approximately 0.5 $L \cdot kg^{-1} \cdot min^{-1}$ and then adjusted throughout the experiment to keep the measured pH in the box within 0.1 unit of 4.4 or 4.8. The water was not recirculated, but rather led to waste after leaving the chamber so as to minimize speciation changes in Al. Total Al and $Ca^{2+}$ levels in the inflowing and outflowing water were monitored daily; values deviated less than 10% from desired.

We avoided most of the complications associated with aging of test solutions, Al precipitation, organic complexation, and pH changes in the bulk water by using freshly prepared solutions which were moved quickly past the fish on a one-pass, flow-through basis. Al speciation can be predicted with some confidence from thermodynamic equilibrium constants for the dissolution of amorphous aluminum hydroxide at known pH and ionic strength (Johnson et al. 1981). At both pH = 4.4 and 4.8, 333 μg/L was well below the total solubility limit. At pH = 4.4, free $Al^{3+}$ constituted about 77% of total Al, and the remainder was mainly $Al(OH)^{2+}$; at pH = 4.8, $Al^{3+}$ was reduced to about 52%, $Al(OH)^{2+}$ was 33%, and $Al(OH)_2^+$ constituted 15%. These pH and Al levels are representative of conditions often seen during snowmelt or rainstorm runoff in acid-sensitive areas of eastern North America and Scandinavia.

### Sampling Regimes

Blood samples were taken from each fish under the acclimation condition (control sample) and at 4, 18, 28, 42, 66, 114, 162, and 240 h after the start of the test condition, if death did not occur earlier. A "terminal sample" refers to the last one taken prior to death. Samples (700 μL) were drawn anaerobically into gas-tight, ice-cold Hamilton syringes via the arterial catheters, without disturbance to the animals, and replaced immediately by reinfusion of Cortland saline. Samples were analyzed for pH, $O_2$ tension, total $CO_2$ (whole blood and true plasma), hematocrit (Ht), hemoglobin (Hb), lactate, glucose, and plasma levels of $Na^+$, $Cl^-$, $K^+$, $Ca^{2+}$, and total protein.

### Analytical Techniques

Arterial blood pH (pHa) and $O_2$ tension ($Pa_{O_2}$) were determined with Radiometer microelectrodes (E5021, E5046) kept

at the experimental temperature and connected to a Radiometer PHM 71 or 72 acid–base analyzer. Total $CO_2$ ($Ca_{CO_2}$) was measured on true plasma and whole blood using either a Corning 965 $CO_2$ analyzer or the Radiometer $CO_2$ microelectrode (E5036) method of Cameron (1971). Plasma was obtained by centrifuging 80 μL of whole blood in heparinized microhematocrit tubes at 5000 × *g* for 5 min. The Ht was read directly from the tube, which was then broken to allow aspiration of the plasma into a Hamilton syringe for transfer to the $CO_2$ analyzer or Cameron chamber. Hb was measured colorimetrically using the cyanmethemoglobin method and Sigma reagents (Blaxhall and Daisley 1973). Lactate was measured enzymatically (L-lactate dehydrogenase/NADH method; Loomis 1961; Sigma reagents) on 100 μL of whole blood which had been immediately deproteinized in 200 μL of ice-cold 8% perchloric acid. Glucose was determined by one of two methods. The colorimetric *O*-toluidine method of Hyvarinon and Nikkita (1962) used 100 μL of whole blood which had been immediately deproteinized in 900 μL of ice-cold 3% trichloroacetic acid. Alternatively, glucose was determined enzymatically on 10 μL of plasma by the hexokinase method of Bondar and Mead (1974). The two methods were cross-validated; both employed Sigma reagents.

The remainder of the blood sample was centrifuged at 9000 × *g* for 2 min to separate plasma. Plasma protein was determined with an American Optical Goldberg refractometer (Alexander and Ingram 1980). Plasma $Na^+$, $K^+$, and $Ca^{2+}$ were appropriately diluted and measured against known standards by atomic absorption spectrophotometry (Varian 1275). $Cl^-$ was determined by coulometric titration (Radiometer CMT10) or by the mercuric thiocyanate spectrophotometric method of Zall et al. (1956) using Sigma reagents; the two methods were cross-validated.

Water total Al levels were routinely monitored by the pyrocatechol violet method of Dougan and Wilson (1974), $Na^+$ and $Ca^{2+}$ by atomic absorption, and pH by a Radiometer GK2401C electrode and PHM82 meter.

### Calculations

Arterial $CO_2$ tension ($Pa_{CO_2}$) was calculated using the Henderson–Hasselbalch equation in the following form:

$$(1)\quad Pa_{CO_2} = \frac{Ca_{CO_2}}{\alpha CO_2 \cdot (1 + \text{antilog}(\text{pHa} - \text{pk}'))}$$

where $Ca_{CO_2}$ was measured in true plasma and values of $\alpha CO_2$ and pK′ at the appropriate temperature were taken from tabulated values for trout plasma (Boutilier et al. 1984). Bicarbonate concentrations in whole blood and true plasma were calculated by

$$(2)\quad [HCO_3^-] = Ca_{CO_2} - (\alpha CO_2 \cdot Pa_{CO_2})$$

using the corresponding measurements of $Ca_{CO_2}$. As plasma $HCO_3^-$ levels were always very similar to (and slightly less than) plasma $Ca_{CO_2}$ levels, only the former have been reported. The concentration of metabolic $H^+$ ($\Delta H^+m$) added to the whole blood was calculated in the cumulative fashion described by McDonald et al. (1980) using the following equation for each interval:

$$(3)\quad [\Delta H^+m] = [HCO_3^-]_1 - [HCO_3^-]_2 - \beta(pHa_1 - pHa_2)$$

and summing (taking account of sign) for each period from the control sample onwards. In this equation, values of $[HCO_3^-]$ are for whole blood, and β represents the nonbicarbonate buffer value for whole blood. The value of β is largely determined by the Hb concentration (Wood et al. 1982). Therefore, we estimated β from the measured [Hb] at each time, using a regression equation derived from in vitro $P_{CO_2}$ equilibration of whole blood from brook trout acclimated to soft water (R. L. Walker and C. E. Booth, unpubl. results):

$$(4)\quad \beta = -0.942[\text{Hb}] - 2.84 \qquad (r = 0.97, N = 5, p < 0.01).$$

Hb-bound $O_2$ content per unit hemoglobin ($[O_2]/[Hb]$) was estimated from the measured $Pa_{O_2}$ and pHa at each time, using a family of $O_2$ dissociation curves determined in vitro for brook trout blood at a range of $P_{CO_2}$'s (R. L. Walker and C. E. Booth, unpubl. results). Mean cell Hb concentration (MCHC, grams Hb per millilitre of red cells) was calculated as the ratio of the Hb and Ht measurements.

### Treatment of Data

Survival curves were compared using standard log/probit analysis and nomographic methods to determine median survival times (LT50), their 95% confidence limits (CL), slope functions (*S*), and the significance of differences between treatments (Litchfield 1949; Litchfield and Wilcoxin 1949). Fish that may have died from factors unassociated with the test condition (e.g. Ht < 6%, bleeding, cannula failure) were excluded from the analysis.

The analysis and presentation of physiological data representative of group responses is complex when individual members of the group die at different times during the test conditions, as was the case in the present study. Simple averaging of all data at a particular time can be very misleading because the fish showing the greatest disturbances are often those which die first; loss of their values from the mean at subsequent sample times gives an incorrect impression of recovery. This problem was avoided in two ways. In the first, means (± 1 SE, *N*) were presented up to the 42-h sample for all fish in a group which survived beyond this time. As 42 h was longer than the LT50 in several test conditions, this analysis showed trends representative of the most resistant fish in the group. In the second approach, the last measurements prior to death ("terminal samples") for each fish in a group were tabulated as means (± 1 SE, *N*), irrespective of when the fish died during the 10-d exposure. These terminal means were compared with the initial means for the same fish during the control period. This analysis illustrated the disturbances which immediately preceded death and therefore more clearly indicated key mechanisms of lethality under different conditions. In both cases, there was a paired design, so Student's two-tailed paired *t*-test was used to compare means back to the control mean for those particular fish. Student's two-tailed unpaired *t*-test was used for comparisons between groups. A 5% significance level was employed throughout.

## Results

### Mortality

Exposure to pH = 4.8, $Ca^{2+}$ = 25 μequiv/L in the absence of Al caused no mortality over 10 d (Fig. 1). Addition of Al (333 μg/L) resulted in >80% mortality with an LT50 (±95% CL) of 39.0 h (29.5–51.5). At higher $Ca^{2+}$, this same exposure (pH = 4.8, $Ca^{2+}$ = 400 μequiv/L, Al = 333 μg/L) caused

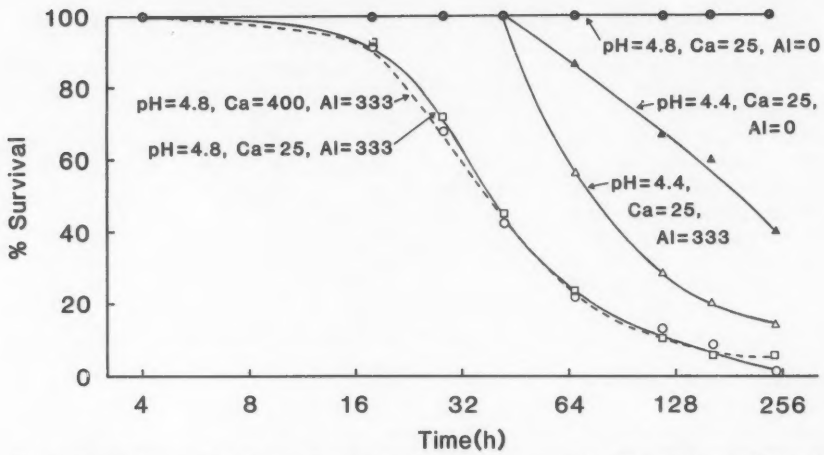


FIG. 1. Survival curves for chronically cannulated brook trout exposed to five different pH/$Ca^{2+}$/Al conditions for a 10-d period. The time scale is logarithmic. $Ca^{2+}$ concentrations are in μequiv/L; Al concentrations are in μg/L.

an almost identical pattern of mortality with unchanged LT50 = 38.5 h (30.3–48.9).

At lower pH (pH = 4.4, $Ca^{2+}$ = 25 μequiv/L), acid exposure alone caused 60% mortality with an LT50 of 170.0 h (103.6–278.8). Addition of Al (333 μg/L) increased mortality to >80% and significantly decreased median survival time to 78.2 h (50.6–120.1). This LT50 was significantly longer than in the same exposure at higher pH (pH = 4.8, $Ca^{2+}$ = 25 μequiv/L, Al = 333 μg/L). There were no significant differences among the slope functions (*S*) of the log time/probit mortality curves in any of the five test conditions.

Relative to the noncannulated fish of Booth et al. (1988), these mortality rates were comparable in the pH = 4.8 tests at low $Ca^{2+}$, but significantly greater at high $Ca^{2+}$ and in the pH = 4.4 tests. This suggests that cannulation and blood sampling may reduce tolerance.

### Effects of Moderate Acidity Alone

Exposure to pH = 4.8, $Ca^{2+}$ = 25 μequiv/L, Al = 0 μg/L had negligible influence on most measured parameters. Major plasma electrolytes (Fig. 2A; Table 1), MCHC (Fig. 3A), and lactate and glucose (Table 2) all remained unchanged throughout 10 d. The only exceptions were a very small but significant drop in pHa (<0.1 unit; Fig. 4A), which was fully corrected by 114 h, a slight rise in plasma $K^+$ (Table 1), and an initial increase in $Pa_{O_2}$ (Fig. 8A). In terms of standard acid–base terminology (Davenport 1974), the acidosis was entirely of "metabolic" origin (Fig. 6A), for there was no "respiratory" component, i.e. no change in $Pa_{CO_2}$ (Fig. 5A). Blood lactate remained constant (Table 2). The rise in $Pa_{O_2}$ (Fig. 8A) had no effect on $[O_2]$/[Hb] (Fig. 9A) because the Hb was already saturated under control conditions.

### Effects of Al in Combination with Moderate Acidity

The presence of Al (333 μg/L), at pH (4.8) and $Ca^{2+}$ levels (25 μequiv/L), which were shown to be nonlethal in the preceding section, killed more than half the fish before the 42-h sample. Ionoregulatory disturbance was prominent, with significant decreases in plasma $Na^+$ and $Cl^-$ appearing by 18 h, even in the most resistant fish (Fig. 2B). In terminal samples, $Na^+$ levels fell to ~109 and $Cl^-$ to ~97 mequiv/L (Fig. 2B), and there was substantial swelling of the red cells as evidenced by the decline in MCHC (Fig. 3B). Acid–base status was little affected (Fig. 4B,5B,6B), but there was a significant rise in $Pa_{CO_2}$ (Fig. 7B) and reciprocal fall in $Pa_{O_2}$ (Fig. 8B) in dying fish. The latter was associated with about a 25% drop in oxygenation of the Hb (Fig. 9B) and a significant rise in blood lactate (Table 2). The rise in blood lactate was also seen in the 42-h survivors (Table 2). While the decline in plasma $Na^+$ and $Cl^-$ appeared to be the most serious of these disturbances, neither this nor the respiratory effects (i.e. changes in $Pa_{O_2}$ and $Pa_{CO_2}$) prior to death were as large as in other test conditions where one or the other mechanism clearly predominated (see below). The results therefore suggest a compound mechanism of toxicity.

### Effects of Higher $Ca^{2+}$ in the Presence of Al at Moderate Acidity

The time course and extent of mortality during exposure to Al (333 μg/L) at pH = 4.8 were unchanged by the presence of higher $Ca^{2+}$ (400 μequiv/L), but there were substantial differences in blood chemistry. The loss of plasma electrolytes was reduced, with terminal levels ($Na^+ \simeq 128$, $Cl^- \simeq 112$ mequiv/L; Fig. 2C) significantly higher than at the same pH and Al levels at low $Ca^{2+}$ (Fig. 2B). However, the respiratory disturbance was much more severe. $Pa_{O_2}$ fell below 30 Torr, even in the most resistant trout (Fig. 8C), and this, together with an accompanying acidosis, lowered $[O_2]$/[Hb] by 50–60% (Fig. 9C). Lactate increased markedly prior to death (Table 2). $Pa_{CO_2}$ more than doubled (Fig. 7C), in mirror image to the fall in $Pa_{O_2}$. This increase in $Pa_{CO_2}$ caused a classic respiratory acidosis, pHa falling by 0.2–0.3 unit (Fig. 4C), while plasma $HCO_3^-$ rose significantly (Fig. 5C). Despite the considerable lactate release (Table 2), there was no metabolic component to the acidosis (Fig. 6C). Other prominent effects were significant increases in plasma $K^+$ and protein concentrations (Table 1) and a red cell swelling which was the most marked of any of the test conditions (Fig. 3C). On balance, these results indicate

TABLE 1. Plasma potassium, calcium, and protein concentrations in 42-h surviving fish (control and 42-h values) and in fish which died at any time (initial and terminal values) during 10 d of exposure to five different pH/[$Ca^{2+}$]/[Al] conditions. Initial values were taken during the control period, and terminal values represent the last measurements prior to death. Means ± 1 SEM.

| Test condition | | | $K^+$ (mequiv/L) | | | | | $Ca^{2+}$ (mequiv/L) | | | | | Plasma Protein (g/100 mL) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 42-h survivors | | Mortalities | | | 42-h survivors | | Mortalities | | | 42-h survivors | | | Mortalities | |
| pH | [$Ca^{2+}$] (μequiv/L) | [Al] (μg/L) | Control | 42 h | N | Initial | Terminal | N | Control | 42 h | N | Initial | Terminal | N | Control | 42 h | N | Initial | Terminal | N |
| 4.8 | 25 | 0 | 2.58 ± 0.08 | 2.92 ± 0.04[a] | 9 | 2.58 ± 0.08 | 2.95 ± 0.14[b] | 9 | 4.57 ± 0.37 | 4.24 ± 0.28 | 9 | 4.57 ± 0.37 | 4.46 ± 0.29[b] | 9 | 3.40 ± 0.30 | 3.28 ± 0.34 | 9 | 3.40 ± 0.30 | 3.60 ± 0.45[b] | 9 |
| 4.8 | 25 | 333 | 3.67 ± 0.63 | 4.80 ± 0.68 | 4 | 3.96 ± 0.36 | 4.64 ± 0.48 | 10 | 4.65 ± 0.55 | 4.56 ± 0.32 | 7 | 4.41 ± 0.15 | 4.47 ± 0.23 | 15 | 4.10 ± 0.28 | 4.10 ± 0.30 | 7 | 3.55 ± 0.19 | 3.79 ± 0.28 | 16 |
| 4.8 | 400 | 333 | 2.72 ± 0.29 | 3.53 ± 0.23[a] | 11 | 2.92 ± 0.18 | 3.83 ± 0.14[a] | 21 | 5.86 ± 0.65 | 5.76 ± 0.45 | 12 | 4.62 ± 0.32 | 5.19 ± 0.22 | 22 | 3.14 ± 0.20 | 3.57 ± 0.32 | 12 | 2.88 ± 0.12 | 3.55 ± 0.20[a] | 24 |
| 4.4 | 25 | 0 | 2.59 ± 0.21 | 2.36 ± 0.26 | 8 | 2.78 ± 0.22 | 2.85 ± 0.36 | 6 | 4.31 ± 0.26 | 4.02 ± 0.15 | 8 | 4.50 ± 0.19 | 3.93 ± 0.38 | 6 | 2.85 ± 0.14 | 2.69 ± 0.16 | 8 | 2.88 ± 0.15 | 3.20 ± 0.38 | 6 |
| 4.4 | 25 | 333 | 3.10 ± 0.39 | 2.74 ± 0.34 | 9 | 3.12 ± 0.59 | 2.62 ± 0.51 | 6 | 4.66 ± 0.21 | 3.98 ± 0.15[a] | 9 | 4.35 ± 0.09 | 3.70 ± 0.15[a] | 6 | 3.01 ± 0.16 | 3.02 ± 0.14 | 9 | 2.75 ± 0.11 | 2.92 ± 0.06 | 6 |

[a]$p < 0.05$ relative to appropriate "control" or "initial" value.
[b]Not terminal when sacrificed after 10 d of exposure.

that respiratory disturbance (i.e. interference with $O_2$ and $CO_2$ exchange), rather than ionic dilution, was the cause of mortality in this test condition.

### Effects of More Severe Acidity Alone

Exposure to pH = 4.4 alone ($Ca^{2+}$ = 25 μequiv/L, Al = 0 μg/L) had more pronounced effects than to pH = 4.8 alone, resulting in 60% mortality over 10 d. Ionoregulatory failure was the prominent feature of the internal disturbance. Plasma $Na^+$ and $Cl^-$ declined progressively, the trend becoming significant after only 4 h of exposure, even in the most resistant fish (Fig. 2D). Terminal ion levels ($Na^+ \simeq 96$, $Cl^- \simeq 85$ mequiv/L) were significantly lower than in any other treatment, and MCHC fell substantially prior to death (Fig. 3D). A classic metabolic acidosis developed, reflected in progressive decreases in pHa (Fig. 4D) and plasma $HCO_3^-$ (Fig. 5D) and an accumulation of metabolic $H^+$ ($\Delta H^+m$) in the blood (Fig. 6D). Lactate increased significantly (Table 2), but the increase was too small to fully account for the $\Delta H^+m$. As $Pa_{CO_2}$ remained unchanged (Fig. 7D), there was no respiratory component to the acidosis. Similarly, $Pa_{O_2}$ (Fig. 8D) and $[O_2]/[Hb]$ (Fig. 9D) were virtually unaffected, even in dying fish; the slight decline in the latter was entirely attributable to acidosis. In summary, these data indicate that under pure acid stress, the toxic mechanism is closely associated with ionoregulatory failure, and perhaps exacerbated by metabolic acidosis. There appears to have been no direct disturbance of respiratory gas exchange.

### Effects of Al in Combination with More Severe Acidity

The presence of Al (333 μg/L) at pH = 4.4, $Ca^{2+}$ = 25 μequiv/L increased mortality and decreased survival time, but the LT50 was significantly longer than under the same $Ca^{2+}$ and Al levels at pH = 4.8. In general the internal changes were similar to these seen with pH = 4.4 alone, but with a superimposed disturbance of $O_2$ and $CO_2$ levels and less ionic dilution prior to death. Decreases in major plasma electrolytes were similar to those at pH = 4.4 alone up to 42 h in surviving trout, but the absolute levels ($Na^+ \simeq 111$, $Cl^- \simeq 97$ mequiv/L) prior to death were not as low (Fig. 2E versus 2D). Indeed, these terminal ion levels were close to those in dying fish at pH = 4.8, $Ca^{2+}$ = 25 μequiv/L, Al = 333 μg/L, where respiratory disturbance was similarly seen. Depressions of pHa (Fig. 4E) and plasma $HCO_3^-$ (Fig. 5E) and elevations of $\Delta H^+m$ (Fig. 6E) reflected a metabolic acidosis similar to that at pH = 4.4 in the absence of Al (cf. Fig. 4D,5D,6D). $O_2$ and $CO_2$ disturbance was negligible in surviving fish up to 42 h, but was clearly seen in the terminal measurements from dying fish. $Pa_{CO_2}$ (Fig. 7E) and lactate (Table 2) rose significantly before death, the former contributing a respiratory component to the acidosis, while $Pa_{O_2}$ (Fig. 8E) and $[O_2]/[Hb]$ (Fig. 9E) fell. On balance, the results suggest a compound mechanism of toxicity involving both ionoregulatory and gas exchange impairment, similar to that seen at pH = 4.8, $Ca^{2+}$ = 25 μequiv/L, Al = 333 μg/L.

## Discussion

### Responses to Acid Stress Alone

*Salvelinus fontinalis* is considered one of the most resistant of the salmonid species to environmental acidity (Grande et al. 1978), a conclusion supported by an extensive toxicological literature (e.g. Menendez 1976; Trojnar 1977; Swarts et al.

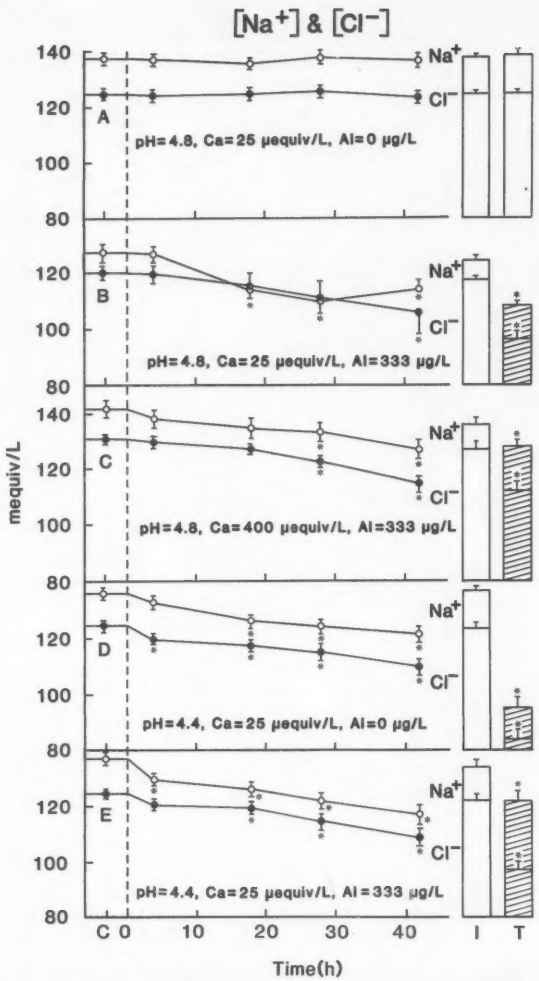


FIG. 2. Changes in plasma $Na^+$ (open circles, upper bar graphs) and $Cl^-$ concentrations (closed circles, lower bar graphs) in chronically cannulated brook trout during 10 d of exposure to five different pH/$Ca^{2+}$/Al conditions in flowing soft water. Data are shown from the control period until 42 h for all fish which survived beyond this time in each test condition (i.e. the most resistant fish in the group). Additionally, terminal data (T; cross-hatched bars) representing the last measurements prior to death in fish dying at any time during the 10 d of exposure are shown in the bar graphs at the right and compared with initial measurements (I; open bars) during the control period for these same fish. In Fig. 2A, none of the fish died, so data taken when the fish were sacrificed at 10 d have been substituted for terminal data. Values are means ± 1 SEM for (A) $N = 9$; (B) $N = 7$ for 42-h survivors, $N = 14$ for mortalities; (C) $N = 12$ for 42-h survivors, $N = 24$ for mortalities; (D) $N = 8$ for 42-h survivors, $N = 6$ for mortalities; and (E) $N = 9$ for 42-h survivors, $N = 6$ for mortalities. Asterisks indicate means significantly different ($p < 0.05$) from the respective control mean (42-h survivors) or initial mean (mortalities) taken during the control period.

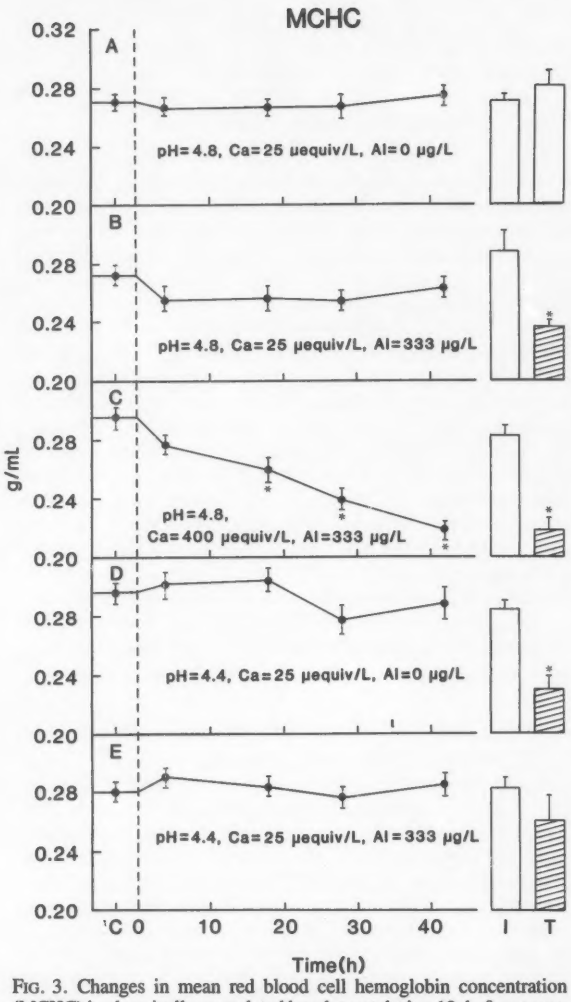


FIG. 3. Changes in mean red blood cell hemoglobin concentration (MCHC) in chronically cannulated brook trout during 10 d of exposure to five different pH/$Ca^{2+}$/Al conditions in flowing soft water. Other details as in legend to Fig. 2.

1978; Schofield and Trojnar 1980; Baker and Schofield 1982). Nevertheless, relevant physiological data on brook trout under acid stress are sparse, limited to studies at pH's below 4.0 and/or involving blood sampling from uncannulated fish in water of unknown composition (Vaala and Mitchell 1970; Mudge and Neff 1971; Dively et al. 1977; Packer 1979). These methodological differences are so great as to render comparison with the present data impractical. However, there exists extensive information on the more sensitive rainbow trout, obtained by comparable cannulation techniques under similar test conditions (cf. Wood and McDonald 1982; McDonald 1983a; Wood 1988 for review).

In the present study, exposure to pH = 4.4 at very low $Ca^{2+}$ (25 μequiv/L) resulted in 60% mortality over 10 d and a severe ionic dilution of the blood plasma in dying brook trout, without evidence of respiratory disturbance. There was also a moderate metabolic acidosis, and a fall in MCHC. Responses to pH = 4.8 without Al were negligible, apart from a small, rapidly corrected acidosis, again of metabolic origin. Ionoregulatory failure as the proximate toxic mechanism in the pH range 4.0–4.5 is entirely consistent with the rainbow trout data (cf. McDonald 1983b; Wood 1988). As in rainbow trout, this effect

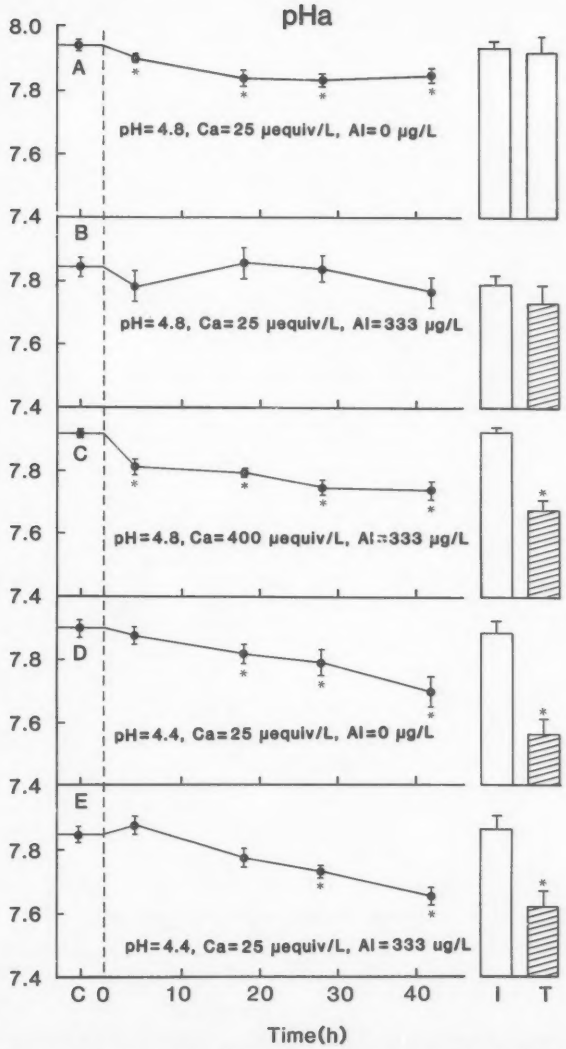


FIG. 4. Changes in arterial blood pH (pHa) in chronically cannulated brook trout during 10 d of exposure to five different pH/$Ca^{2+}$/Al conditions in flowing soft water. Other details as in legend to Fig. 2.

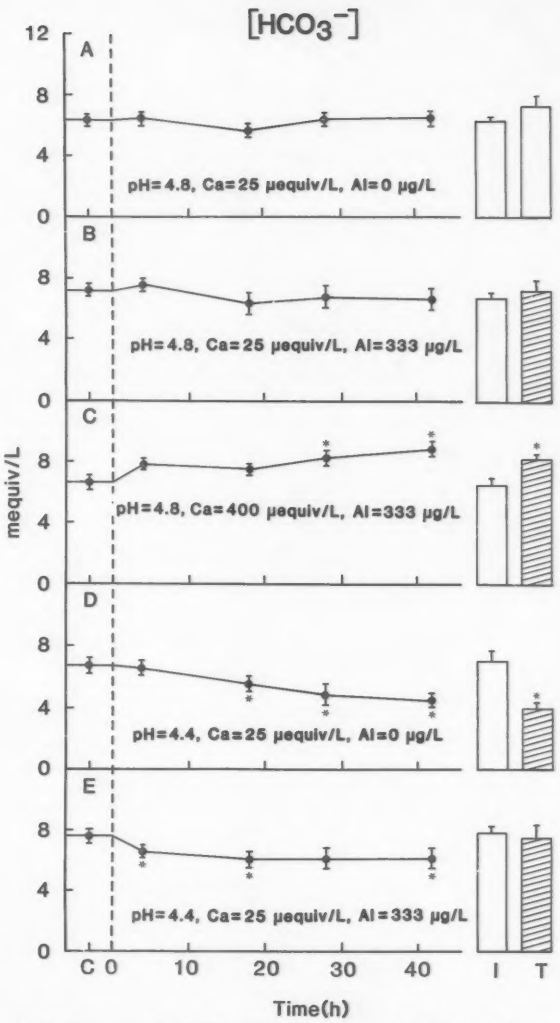


FIG. 5. Changes in the $HCO_3^-$ concentration of arterial blood plasma in chronically cannulated brook trout during 10 d of exposure to five different pH/$Ca^{2+}$/Al conditions in flowing soft water. Other details as in legend to Fig. 2.

was due to a stimulation of passive $Na^+$ and $Cl^-$ efflux across the gills and a more persistent inhibition of active influx, both of which were demonstrated in the preceding study (Booth et al. 1988). These results agree with the early work of Packer and Dunson (1970, 1972) on brook trout. The decrease in MCHC (Fig. 3D) was also similar to the rainbow trout response, resulting from erythrocytic swelling in response to ionic dilution of the plasma, as discussed by Milligan and Wood (1982). However, the occurrence of a moderate metabolic acidosis was surprising because rainbow trout exhibit this response only in acidified hard water. In acidified soft water, similar to the present test conditions, they show virtually unchanged pHa because at low $Ca^{2+}$ levels the net losses of $Na^+$ and $Cl^-$ are equal, preventing net $H^+$ "entry" (McDonald 1983b; Wood 1988). The relationship between water $Ca^{2+}$ and net $Na^+$ and $Cl^-$ losses is presumably different in brook trout.

There was no blood glucose response during acid exposure or any other test condition of the present study, even in dying fish (Table 2). Its absence may be related to the pre-experimental starvation period. Glucose mobilization is considered a general "stress" response in fish, as well as serving an osmoeffector function during acid exposure in rainbow trout (McDonald 1983b; Brown et al. 1984). Plasma $Ca^{2+}$ levels were also generally unresponsive (Table 1), but this was in accord with rainbow trout data (McDonald et al. 1980). Plasma $K^+$ concentrations tended to increase (Table 1) despite increased branchial losses in many of the treatments (Booth et al. 1988). This has been commonly observed in acid-stressed rainbow trout and reflects both hemoconcentration and an efflux of $K^+$ from the intracellular compartment of white muscle (McDonald et al. 1980; Wood and McDonald 1982).

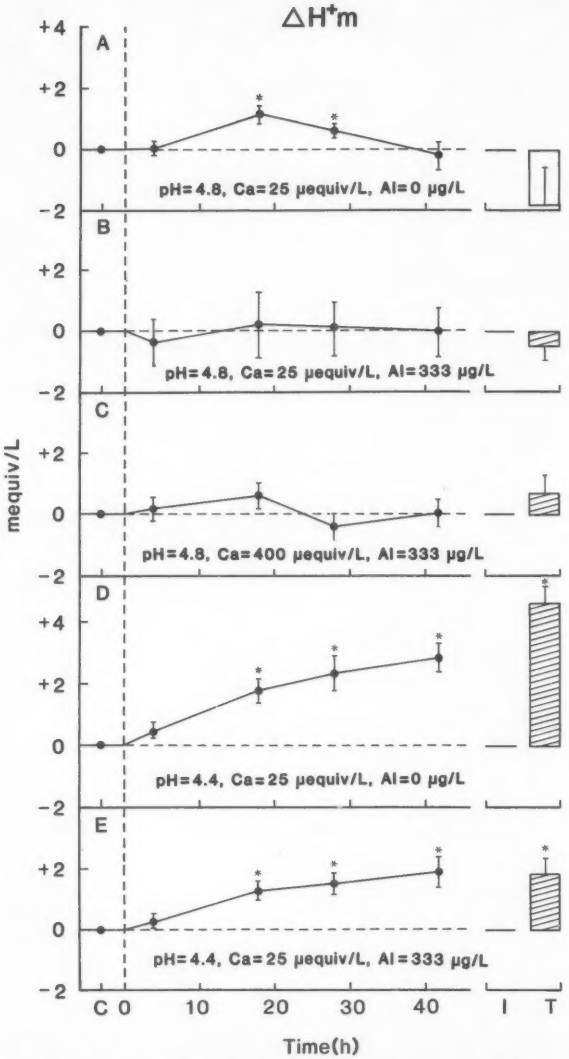


FIG. 6. Changes in the calculated metabolic acid load ($\Delta H^+m$) in whole blood of chronically cannulated brook trout during 10 d of exposure to five different pH/$Ca^{2+}$/Al conditions in flowing soft water. By definition, $\Delta H^+m$ is zero in the control or initial samples. Other detals as in legend to Fig. 2.

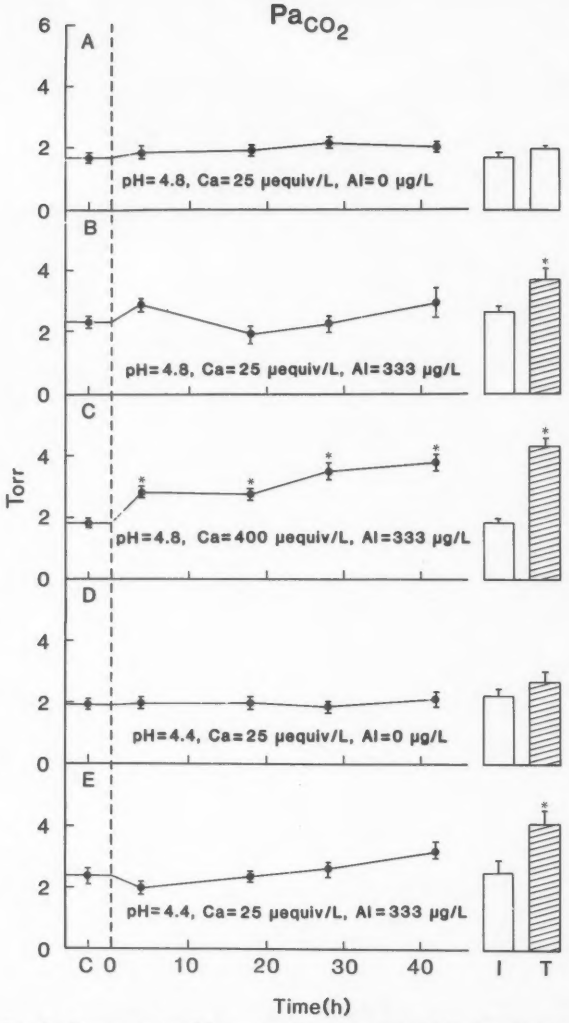


FIG. 7. Changes in the $CO_2$ tension ($Pa_{CO_2}$) of arterial blood in chronically cannulated brook trout during 10 d of exposure to five different pH/$Ca^{2+}$/Al conditions in flowing soft water. Other details as in legend to Fig. 2.

## Responses to Acid plus Al Stress

The presence of Al clearly increased toxicity relative to acid alone at both pH = 4.8 and 4.4 at very low $Ca^{2+}$ (25 μequiv/L). In terms of mortality and LT50, this effect was much more dramatic at pH = 4.8 (Fig. 1), but changes in blood chemistry were similar at the two pH's. Ionoregulatory failure occurred at both pH's in the presence of Al (while it did not at pH = 4.8 in the absence of Al), but the terminal plasma $Na^+$ and $Cl^-$ levels in dying fish were not as low as at pH = 4.4 in the absence of Al (Fig. 2). There are two related reasons for this difference.

Firstly, in the presence of Al, the fish died more quickly (Fig. 1) and lost $Na^+$ and $Cl^-$ more rapidly to the water, as demonstrated in the preceding flux study (Booth et al. 1988). This suggests that the rate, rather than the absolute loss, may be the more important factor involved in lethality, probably because of the resultant internal fluid shifts and circulatory collapse accompanying high $Na^+$ and $Cl^-$ efflux rates (Milligan and Wood 1982; McDonald 1983b; Wood 1988). At pH = 4.4 in the absence of Al, the fish lost $Na^+$ and $Cl^-$ more slowly and therefore could withstand lower absolute concentrations of these ions in the blood plasma prior to death.

Secondly, pure ionoregulatory failure was not the sole cause of death, for there was also clear evidence of disturbance to respiratory gas exchange. This was reflected in elevations of $Pa_{CO_2}$ (Fig. 7B,7E) and lactate (Table 2) and depressions of $Pa_{O_2}$ (Fig. 8B,8E) which resulted in 25–35% decreases in the $O_2$ saturation of the Hb (Fig. 9B,9E). Respiratory problems probably accelerated the death of the fish, so again, plasma ion

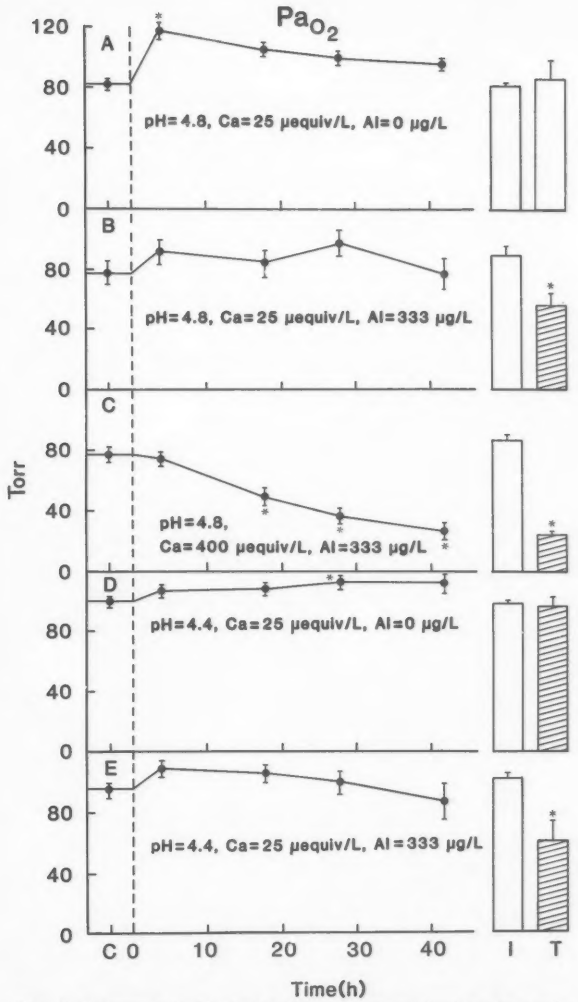


FIG. 8. Changes in the $O_2$ tension ($Pa_{O_2}$) of arterial blood in chronically cannulated brook trout during 10 d of exposure to five different pH/$Ca^{2+}$/Al conditions in flowing soft water. Other details as in legend to Fig. 2.

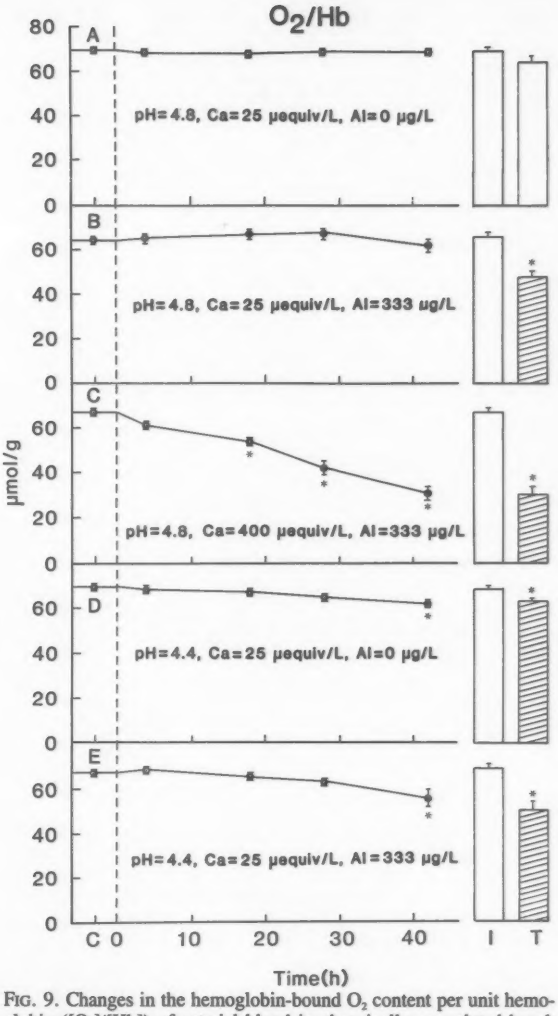


FIG. 9. Changes in the hemoglobin-bound $O_2$ content per unit hemoglobin ($[O_2]/[Hb]$) of arterial blood in chronically cannulated brook trout during 10 d of exposure to five different pH/$Ca^{2+}$/Al conditions in flowing soft water. Other details as in legend to Fig. 2.

levels did not fall as low as when fish died more slowly from pure ionoregulatory failure.

The present finding that Al exacerbates acid toxicity at both pH = 4.4 and 4.8, but to a greater extent at the higher pH, is in general accord with previous toxicological studies on brook trout (Schofield and Trojnar 1980; Baker and Schofield 1982). There have been no previous physiological studies on brook trout under combined acid plus Al stress, but several studies have been done on other salmonids (Muniz and Leivestad 1980a, 1980b; Leivestad et al. 1980; Rosseland 1980; Neville 1985; Rosseland et al. 1986; Witters 1986). While none of these studies are directly comparable, there does seem to be general agreement that Al causes both ionoregulatory and respiratory disturbances and that these effects are more intense at higher (but still acidic) pH's, in accord with the present data. The most detailed of these investigations, that of Neville (1985) on rainbow trout, concluded that the fish died from electrolyte loss at pH = 4.0–4.5 but from hypoxia at pH = 6.1, with a transition in causation between these levels. Taking into account the greater tolerance of brook trout for low pH, our data do not conflict with Neville's (1985) interpretation.

In the preceding study (Booth et al. 1988), we have shown that the effect of Al on branchial ion balance, over and above that due to acidity alone, was primarily increased efflux of $Na^+$ and $Cl^-$, combined with a small further inhibition of the active uptake of these ions. The effect was greater at pH = 4.8 than at 4.4 and was associated with the accumulation of Al on the gills; dying fish showed the greatest branchial Al burdens. Other studies have demonstrated branchial mucification, edema, and even lamellar fusion in Al-exposed fish (Muniz and Leivestad 1980a, 1980b; Schofield and Trojnar 1980; Tandjung 1982; Karlsson-Norrgren et al. 1986). A present working hypothesis is that precipitation of aluminum hydroxides and/or binding of Al to organic anions occurs on the gill surface, inducing an inflammatory response. In turn, this stimulates mucus produc-

TABLE 2. Blood lactate and glucose concentrations in 42-h surviving fish (control and 42-h values) and in fish which died at any time (initial and terminal values) during 10 d of exposure to five different pH/[$Ca^{2+}$]/[Al] conditions. Initial values were taken during the control period, and terminal values represent the last measurements prior to death. Means ± 1 SEM.

| Test condition | | | Lactate (mequiv/L) | | | | | | Glucose (mmol/L) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 42-h survivors | | | Mortalities | | | 42-h survivors | | | Mortalities | | |
| pH | [$Ca^{2+}$] (μequiv/L) | [Al] (μg/L) | Control | 42 h | *N* | Initial | Terminal | *N* | Control | 42 h | *N* | Initial | Terminal | *N* |
| 4.8 | 25 | 0 | 0.48 ±0.06 | 0.79 ±0.17 | 9 | 0.48 ±0.06 | 1.07 ±0.42[b] | 9 | 3.84 ±0.76 | 2.91 ±0.47[b] | 9 | 3.84 ±0.76 | 3.18 ±0.34[b] | 9 |
| 4.8 | 25 | 333 | 1.10 ±0.26 | 2.39 ±0.69[a] | 6 | 1.15 ±0.15 | 3.63 ±0.92[a] | 14 | 7.96 ±1.37 | 11.46 ±2.35 | 7 | 8.22 ±0.99 | 7.57 ±1.20 | 16 |
| 4.8 | 400 | 333 | 0.67 ±0.27 | 2.86 ±0.73[a] | 12 | 0.77 ±0.13 | 5.04 ±0.69[a] | 22 | 5.64 ±0.49 | 5.83 ±1.01 | 12 | 5.75 ±0.32 | 5.57 ±0.55 | 22 |
| 4.4 | 25 | 0 | 0.70 ±0.09 | 1.45 ±0.31[a] | 8 | 0.68 ±0.15 | 1.74 ±0.43[a] | 5 | 8.13 ±1.17 | 5.78 ±0.87 | 12 | 8.20 ±1.32 | 11.95 ±4.10 | 6 |
| 4.4 | 25 | 333 | 0.60 ±0.11 | 1.52 ±0.66 | 9 | 0.68 ±0.16 | 2.25 ±0.90[a] | 6 | 6.61 ±0.70 | 5.08 ±0.71 | 9 | 7.35 ±0.91 | 6.88 ±1.58 | 6 |

[a]$P < 0.05$ relative to appropriate "control" or "initial" value.
[b]Not terminal when sacrificed after 10 d of exposure.

tion, thickens and distorts the branchial epithelium, decreases its transcellular permeability to $O_2$ and $CO_2$, yet simultaneously increases the permeability of paracellular channels through which the majority of electrolyte loss is thought to occur (McDonald 1983a; Wood 1988). At low water pH's, the lamellar microenvironment is undoubtedly less acidic than the bulk medium, due mainly to $NH_3$ efflux (Wright and Wood 1985). As gill water flow encounters this more basic milieu, the formation of aluminum hydroxide complexes will be favoured. Depending on the actual pH shift and Al concentration, supersaturating conditions may occur, resulting in direct precipitation of Al on the gill. This interpretation would explain the greater toxic effect of Al at pH = 4.8 than at 4.4, for at the latter pH, solubility is higher and aluminum hydroxide concentrations are lower. Alternatively, the greater toxicity at pH = 4.8 may reflect a greater reactvity of cationic aluminum hydroxides than $Al^{3+}$ with surface binding sites. While this interpretation views precipitation as secondary to binding, the two explanations are certainly not mutually exclusive.

Fish kills in the field may occur at pH's greater than 4.8 (Dickson 1983; Henriksen et al. 1984), and field surveys suggest that loss of fish populations is progressive as pH's fall from 6.5 to 5.0 (Magnuson et al. 1984). Neville (1985) found severe physiological responses in rainbow trout exposed to low levels of Al at pH = 6.1, responses which lessened at both 6.5 and 5.0. The chemistry and solubility of Al are poorly understood at these higher pH's, and there is little experimental information on fish responses. There is a clear need for physiological, toxicological, and water chemistry studies with Al in this ecologically significant pH range (4.8–6.1).

In the present study, we did not test the effects of Al at circumneutral pH (i.e. Al in the absence of acid stress) because our objective was to evaluate the effects of different pH and $Ca^{2+}$ levels on the responses to a constant level of *dissolved* Al (333 μg/L). Al solubility is extremely low at circumneutral pH and would have been greatly exceeded by Al = 333 μg/L, resulting in unstable, supersaturated solutions with suspended particles (Johnson et al. 1981; Neville 1985).

### Influence of Higher $Ca^{2+}$ on the Responses to Acid plus Al Stress

At higher water $Ca^{2+}$ (400 μequiv/L), a level still well within the softwater range, the physiological response to Al = 333 μg/L at pH = 4.8 was fundamentally altered to one where respiratory disturbances clearly predominated. Ionoregulatory effects were greatly attenuated (Fig. 2C). $Pa_{O_2}$ fell precipitously (Fig. 8C) and $Pa_{CO_2}$ rose reciprocally (Fig. 7C) to levels representative of *venous* blood in cannulated brook trout (Walker et al. 1988). These changes resulted in respiratory acidosis (Fig. 4C,5C), arterial $O_2$ saturations less than half the control levels (Fig. 9C), and substantial lactate release (Table 2). The rise in $Pa_{CO_2}$ undoubtedly contributed to the marked drop in MCHC (Fig. 3C), for this is known to potentiate erythrocytic swelling (Wood et al. 1982).

The protective effect of $Ca^{2+}$ on ion balance agrees with the results of the flux experiments (Booth et al. 1988). It also agrees with general theory (McDonald 1983a; McDonald et al. 1988), for $Ca^{2+}$ is known to reduce paracellular channel permeability and to compete with metals for surface ligands. Elevations in $Ca^{2+}$ across a comparable range similarly attenuated plasma $Na^+$ and $Cl^-$ depressions in Al-exposed brown trout (*Salmo trutta*) (Muniz and Leivestad 1980a, 1980b) but not in Al-exposed rainbow trout (Witters 1986). However, this discrepancy is probably due to the acutely low pH (4.1) and short duration (3.5 h) of the tests in the latter study.

The exacerbating effect of higher $Ca^{2+}$ on respiratory failure has not been reported previously. It presumably explains why ion loss rates did not predict mortality in the same way as at low $Ca^{2+}$ and why there was no clear relationship between pH and ion losses in the presence of Al at $Ca^{2+}$ = 400 μequiv/L (Booth et al. 1988). This toxic effect at the gill surface must in some way reflect a greater increase in resistance to $O_2$ and $CO_2$ diffusion, but we can only speculate as to mechanism. Possibilities include a greater inflammatory response, more mucus production, a promotion of Al polymerization or precipitation by $Ca^{2+}$, and more Al-binding sites in fish acclimated and

exposed at higher water $Ca_{2+}$. However, we could detect no difference in net branchial accumulation of Al between $Ca^{2+}$ = 25 and 400 μequiv/L treatments (Booth et al. 1988). The problem clearly requires detailed morphological study.

The observation of respiratory toxicity at higher $Ca^{2+}$ concentration may have considerable environmental significance. A number of fish kills in the wild associated with ameliorative liming of lakes and streams have been reported (e.g. Dickson 1978, 1983). Liming increases both the water $Ca^{2+}$ level and pH without altering total Al, at least in the short term. The present observations and the data of Neville (1985), when taken together, suggest that increases in $Ca^{2+}$ and pH should both promote respiratory failure, while simultaneously reducing ionoregulatory disturbance. Thus under some circumstances, liming may simply exchange one toxic effect of environmental acidity and Al for another.

## Acknowledgements

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# Physiological Evidence of Acclimation to Acid/Aluminum Stress in Adult Brook Trout (*Salvelinus fontinalis*). 1. Blood Composition and Net Sodium Fluxes

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Brook trout (*Salvelinus fontinalis*) adapt to chronic sublethal acid/Al stress. The accompanying acclimation confers greater resistance to short-term increases in Al and acidity. Adult trout were exposed in flowing soft water to eight combinations of pH (6.5, 5.2) × $Ca^{2+}$ (25, 400 μequiv/L) × Al (0, 75, 150 μg/L = 0, 2.8, 5.6 μmol/L). After 10 wk, blood sampling by caudal puncture revealed no significant variations in osmolality, plasma protein, or hemoglobin and only minor differences (≤15%) in plasma $Na^+$ and $Cl^-$. Overall, most electrolytes were higher in fish exposed to higher water Al and/or $Ca^{2+}$; only plasma $Ca^{2+}$ was directly depressed by low pH. Hematocrit was raised by both low pH and elevated Al. When trout naive to both acid and Al were challenged with pH = 4.8, Al = 333 μg/L under flow-through conditions, there were large negative whole-body $Na^+$ fluxes and marked depressions of plasma $Na^+$ and $Cl^-$, hemoconcentration, and substantial mortality over 48 h. Prior exposure for 10 wk to pH = 5.2 plus either 75 or 150 μg Al/L prevented mortality and ameliorated or abolished these effects through a more rapid recovery of net $Na^+$ balance. Prior exposure to pH = 5.2 alone ameliorated these effects only slightly.

L'omble de fontaine (*Salvelinus fontinalis*) s'adapte à un stress chronique entraîné par des teneurs sublétales en acide et en Al; l'accoutumance concomitante lui confère une plus grande résistance à des augmentations à court terme de la teneur en Al et de l'acidité. On a exposé des ombles adultes à huit différentes combinaisons de pH (6,5 et 5,2), de teneurs en $Ca^{+2}$ (25 et 400 μequiv/L) et de teneurs en Al (0, 75 et 150 μg/L = 0, 2,8 et 5,6 μmol/L) en eau douce à débit continu. Après 10 sem, l'échantillonnage du sang par ponction caudale n'a pas révélé de variations significatives de l'osmolalité, des niveaux de protéine dans le plasma ou du taux de l'hémoglobine, mais on a observé de légères différences (≤15 %) des teneurs en $Na^+$ et $Cl^-$ du plasma. En général, les niveaux de la plupart des électrolytes étaient plus élevés chez les poissons exposés à des teneurs élevées en Al et/ou en $Ca^{+2}$ en milieu aqueux; seule la teneur en $Ca^{+2}$ du plasma a été directement abaissée par un faible pH. Un faible pH et une teneur élevée en Al ont tous deux entraîné une augmentation du taux de l'hématocrite. Chez des ombles jamais exposés à un milieu acide ajouté d'Al et provoqués dans un milieu de pH 4,8 et de teneur en Al de 333 μg/L en débit continu, on a observé d'importants flux négatifs de la teneur en $Na^+$ dans tout l'organisme et des baisses marquées de la teneur en $Na^+$ et $Cl^-$ du plasma et de l'hémoconcentration ainsi qu'une importante mortalité échelonnée sur 48 h. Une exposition préalable pendant 10 sem au pH 5,2 en présence de 75 ou 150 μgAl/L a prévenu cette mortalité et a amélioré ou éliminé ces effets par suite du rétablissement plus rapide de l'équilibre net du $Na^+$. L'exposition préalable au pH 5,2 n'a que légèrement atténué ces effets.

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Rapid increases in dissolved aluminum (Al) to 200–1000 μg/L may accompany episodic depressions in pH during runoff from snowmelt and rainstorms (Christopherson et al. 1984; Marmorek et al. 1985). These Al elevations are widely believed to contribute to acute toxicity observed in the field. Lethal effects of Al on fish have been well documented in the laboratory at these levels (e.g. Baker and Schofield 1982; Brown 1983), and some progress has been made in understanding their physiological basis (e.g. Leivestad 1982; Neville 1985).

However, it is less widely appreciated that fish are now chronically exposed to lower, but nevertheless potentially harmful, background levels of Al in natural soft waters in many acid-sensitive regions of the world. For example, Wright and Snekvik (1978) found Al at 50–300 μg/L in over 90% of 700 lakes in southern Norway, while Schofield and Trojnar (1980) reported a mean Al of 111 μg/L in 28 lakes with surviving brook trout (*Salvelinus fontinalis*) populations in the northeastern United States. A recent survey of 810 lakes in eastern Canada, many with reproducing brook trout populations, has reported a mean Al of 84 μg/L (Kelso et al. 1986). The mean lake pH was acidic (4.5–6.0) in all of these examples. These figures are for total Al; in the absence of detailed water chemistry data, it is difficult to estimate the size of the monomeric fraction and its toxicity to fish. Nevertheless, there is considerable evidence that total Al concentrations of this magnitude can exert damaging sublethal or even lethal effects on fish which have not been exposed previously to Al (e.g. Baker and Schofield 1982; Neville 1985). Therefore, fish chronically exposed to low levels of Al and acidity in the wild may have become acclimated, increasing their tolerance to these and more toxic levels. Alternatively, they may have become weakened and sensitized by these low-level exposures.

Laboratory evidence indicates that acclimation to Al may occur. Orr et al. (1986) demonstrated increased resistance to Al by rainbow trout (*Salmo gairdneri*) exposed for 7–21 d to Al at 50% of the LC50 (at constant pH $\simeq$ 5.2). In brook trout, Siddens et al. (1986) reported greater survival over a 24-d period at pH = 4.9 if a given maximum level of Al was presented continuously, rather than intermittently, which can be interpreted as indirect evidence of acclimation. However, nothing is known about the physiological changes which might occur during acclimation.

The overall objectives of the present and following two papers (Wood et al. 1988b; McDonald and Milligan 1988) were to use physiological criteria, especially ionoregulatory parameters, to (i) confirm that adult brook trout adapt to sublethal Al at pH = 5.2, (ii) assess whether such adaptation confers acclimation, i.e. greater resistance to simultaneous increases in both Al and acidity as would be seen during episodic acid surges in the field, (iii) assess whether prior exposure to acidity alone also confers increased resistance to such surges, and (iv) understand the mechanisms involved in any such increased resistance. This first paper focusses on physiological changes after prolonged exposure (10 wk) to sublethal acid or acid/Al levels, and whole-body $Na^+$ fluxes of these same fish during an acid/Al challenge (pH = 4.8, Al = 333 μg/L) which is in the lethal range for naive fish (cf. Booth et al. 1988). The possible influence of water $Ca^{2+}$ on these processes was assessed using levels representative of the upper and lower ranges of natural soft water. As far as possible, test conditions and methods duplicated those used in our companion studies on naive brook trout (Booth et al. 1988; Wood et al. 1988a).

## Materials and Methods

### Experimental Animals and Holding Conditions

Experiments were conducted at the Fish Physiology and Toxicology Laboratory, Laramie, Wyoming (altitude = 2200 m), which provided facilities for continuous long-term exposures of adult fish to defined pH, $Ca^{2+}$, and Al conditions in flowing artificial soft water. The overall experimental plan was to first acclimate the fish to soft water and then to expose separate groups to a variety of pH, $Ca^{2+}$, and Al combinations for 10 wk. At the end of this period, blood was sampled from a subset of the fish in each group to evaluate the effects of this long-term sublethal exposure on ionic and hematological parameters. Other fish from selected groups were then challenged with a higher Al/lower pH condition (potentially lethal) for 48 h; $Na^+$ flux rates, mortality, and final blood parameters were measured to assess resistance to this more severe challenge.

Artificial soft water was generated by treatment of hard well water (composition in Table 1) with sediment filtration, NaCl softening, reverse osmosis, and separate bed deionization (water system designed by Continental Water Systems, Denver, Colorado). The pH was adjusted to 6.5 with KOH and the water was thoroughly mixed and then delivered to individual head tanks, where the pH was lowered to the desired level with $H_2SO_4$. The carbonate alkalinity of this water at pH = 6.5 was about 2.5 mg/L. All pH adjustments were made with Leeds and Northrup pH controllers (pH analyzer/controller model No. 7083). Lastly, the required levels of $CaCl_2$ and $AlCl_3$ were added via Mariotte bottles to continuous-flow serial diluters, from where the water was delivered to the exposure tanks, and then to waste. The tanks were round 340-L fiberglass chambers served with a flow of 1.9 L/min, providing 8 volume additions per day. Each tank contained 25 fish and represented one of the eight pH/$Ca^{2+}$/Al combinations which were tested (Table 1). Two tanks (50 fish) were also run with hard well water, which differed principally in its higher $Ca^{2+}$ and pH levels (Table 1).

Test animals were 18-mo-old brook trout (mean weight $\simeq$170 g), obtained from Cline's Trout Farm (Boulder, Colorado) and initially acclimated for 34 d to artificial soft water (pH = 6.5, $Ca^{2+}$ = 25 μequiv/L). The exposure period started on May 15, 1985, and was approximately 10 wk (actual range 64–85 d). The pH was gradually reduced from acclimation (6.5) to the desired level over 3 d, and then, Al addition was started 6 d later (day 0 of exposure). To prevent mortality, the set pH in the acidic treatments was raised from 5.0 to 5.2 on day 16. Detailed growth data were recorded on all fish and are reported in Mount et al. (1988).

Experimental temperature was 11 ± 1°C, conductivity ranged from 40 to 115 μS/cm, depending on the particular pH/$Ca^{2+}$/Al combination, and dissolved $O_2$ was maintained above 60% saturation. Fish were fed 1% body weight/day floating trout chow (Purina No. 5106) and tanks were siphoned daily. Photoperiod was adjusted biweekly to follow the natural cycle for Laramie, Wyoming. Water pH was measured daily (Orion Ross combination electrode No. 810200 on a Sargent Welch LS pH meter) and other parameters weekly (Al, $Ca^{2+}$, $Na^+$, $Cl^-$, $SO_4^{2-}$) on samples taken directly from the tanks.

### Blood Sampling

On day 68, six fish were selected at random for blood sampling from each of the nine exposure conditions (eight pH/$Ca^{2+}$/

TABLE 1. Intended exposure conditions, measured water chemistry (means and ranges for weekly me

| Intended | | | | | Al (μg/L) | |
|---|---|---|---|---|---|---|
| pH | $Ca^{2+}$ (μequiv/L) | Al (μg/L) | pH | $Ca^{2+}$ (μequiv/L) | Total | Monomeric |
| 6.5 | 400 | 0 | 6.35 ± 0.03 | 342 (330 – 349) | 4 (<1 – 7) | 3 (< 1 – 5) |
| 6.5 | 25 | 0 | 6.48 ± 0.03 | 28 (20 – 41) | 4 (<1 – 9) | 4 (< 1 – 6) |
| 5.2[a] | 400 | 0 | 5.19 ± 0.05[a] | 337 (308 – 365) | 3 (< 1 – 6) | 3 (< 1 – 6) |
| 5.2[a] | 25 | 0 | 5.25 ± 0.06[a] | 29 (24 – 38) | 3 (<1 – 7) | 3 (<1 – 7) |
| 5.2[a] | 400 | 75 | 5.12 ± 0.05[a] | 341 (313 – 375) | 81 (62 – 117) | 73 (58 – 84) |
| 5.2[a] | 25 | 75 | 4.95 ± 0.05[a] | 26 (21 – 39) | 77 (61 – 93) | 89 (75 – 103) |
| 5.2[a] | 400 | 150 | 5.13 ± 0.05[a] | 339 (315 – 364) | 136 (126 – 146) | 134 (115 – 146) |
| 5.2[a] | 25 | 150 | 5.07 ± 0.06[a] | 29 (22 – 42) | 156 (139 – 187) | 147 (133 – 157) |
| Hard water | | | 7.8 ± 0.01 | 2550 (2440 – 2680) | 2 (<1 – 4) | 1 (<1 – 5) |

[a]Intended pH was originally 5.0 but was raised to 5.2 on day 16. The means over the entire exposure p
[b]% mortality out of 25 fish in each treatment, except in hard water, where there were 50 fish.

ekly measurements), and total mortality during the 10-wk exposure period.

| Measured | | | | | |
|---|---|---|---|---|---|
| µg/L) | | | | | |
| omeric | Inorganic monomeric | $Na^+$ (µequiv/L) | $Cl^-$ (µequiv/L) | $SO_4^{2-}$ (µequiv/L) | Mortality (%)[b] |
| 3 | 3 | 222 | 431 | 47 | 0 |
| 1 – 5) | (< 1 – 7) | (73 – 453) | (400 – 451) | (16 – 106) | |
| 4 | 4 | 213 | 93 | 44 | 0 |
| 1 – 6) | (< 1 – 8) | (75 – 433) | (90 – 96) | (<10 – 106) | |
| 3 | 1 | 223 | 437 | 106 | 0 |
| 1 – 6) | (<1 – 2) | (70 – 466) | (408 – 490) | (13 – 206) | |
| 3 | 2 | 215 | 90 | 102 | 4 |
| 1 – 7) | (<1 – 4) | (71 – 452) | (76 – 99) | (10 – 202) | |
| 73 | 21 | 222 | 439 | 106 | 0 |
| – 84) | (10 – 40) | (64 – 469) | (411 – 493) | (14 – 206) | |
| 89 | 17 | 226 | 104 | 105 | 28 |
| – 103) | (10 – 26) | (73 – 457) | (96 – 113) | (19 – 202) | |
| 34 | 62 | 222 | 470 | 153 | 0 |
| – 146) | (33 – 92) | (70 – 470) | (437 – 504) | (99 – 206) | |
| 47 | 48 | 221 | 112 | 108 | 16 |
| – 157) | (20 – 87) | (71 – 460) | (107 – 115) | (19 – 206) | |
| 1 | 4 | 334 | 275 | 507 | 0 |
| 1 – 5) | (<1 – 7) | (279 – 391) | (253 – 301) | (480 – 542) | |

xposure period are reported for 60 daily measurements ± 1 SE.
sh.

Al combinations plus hard water). Each fish was netted individually into a bucket containing 40 mg MS-222/L (Sigma) to induce light anaesthesia. After 3 min, a 500-μL blood sample was rapidly withdrawn into a heparinized syringe (rinsed with 500 IU ammonium heparin/L (Sigma) by blind puncture of the caudal vein. Fish were returned to their holding tanks for a minimum 1-wk recovery prior to use in any other experiments (Wood et al. 1988b; McDonald and Milligan 1988). Blood samples were analyzed for hematocrit (Ht), hemoglobin (Hb), and plasma levels of $Na^+$, $Cl^-$, $K^+$, $Ca^{2+}$, osmolality, and total protein.

At the end of the 48-h challenge experiments (see below), blood from surviving fish was sampled as described above, except that the anaesthetic was added directly to the closed flux box to minimize disturbance. These blood samples were analyzed for Ht and plasma levels of $Na^+$, $Cl^-$, and total protein. Thus, these parameters can be compared before and after the 48-h challenges for fish in each of the eight pH/$Ca^{2+}$/Al exposure conditions.

## Flux and Challenge Experiments

Between days 64 and 85, six fish from each of the eight pH/$Ca^{2+}$/Al exposure conditions were transferred to individual flux boxes (cf. Booth et al. 1988) which received a continuous flow (~0.5 $L \cdot kg^{-1} \cdot min^{-1}$) of water *identical in composition to that of their exposure condition*. Water $P_{O_2}$ was maintained above 115 Torr, $P_{CO_2}$ levels below 1 Torr, and $HCO_3^-$ levels ranged from 50 μequiv/L at pH = 6.5 to 0 at pH = 4.8. After a 24-h settling period, the flow rate and inflow pH were adjusted to maximize detection of inflow versus outflow differences in $Na^+$ while simultaneously maintaining the measured pH in the box within 0.05 unit of the intended value. After a further 4–6 h, water samples were taken for measurement of $Na^+$ flux rates by the Fick principle (cf. Booth et al. 1988). These measurements represented net $Na^+$ exchange under the exposure condition and therefore were the *control* values for the subsequent challenges. The inflowing water was then changed to the challenge condition.

The challenge condition was pH = 4.8, Al = 333 μg/L at the same $Ca^{2+}$ (25 or 400 μequiv/L) as that in the exposure condition; this combination was previously shown to be in lethal range for naive brook trout (Booth et al. 1988). In addition, six fish from each of the zero-Al exposure conditions (pH = 6.5, $Ca^{2+}$ = 25 or 400 μequiv/L) were challenged with pH = 4.8, Al = 0 μg/L to discern the effect of acidity alone. $Na^+$ flux measurements were taken at 4, 8, 12, 24, 30, 36, and 48 h during all challenges. Immediately after the 48-h determinations, blood samples were drawn from surviving fish as described above. Inflow pH and flow rate were adjusted throughout the experiment to maintain the desired conditions. Water was not recirculated but, rather, led to waste after leaving the chambers to minimize speciation changes in Al. Al and $Ca^{2+}$ levels were monitored daily in inflowing and outflowing water; deviations from intended values were less than 20%.

## Analytical Techniques and Calculations

Analytical methods were identical to those described in Booth et al. (1988) and Wood et al. (1988a) with the following exceptions. Plasma $Na^+$ and water $Na^+$ and $Ca^{2+}$ levels were determined on a Perkin–Elmer model No. 2380 atomic absorption spectrophotometer. Water Al was measured with an associated high-temperature graphite furnace (Perkin–Elmer model No. HGA-400). Al was speciated into total, monomeric, and inorganic monomeric after the method of LaZerte (1984). Water $Cl^-$ and $SO_4^{2-}$ levels were measured using a Dionex ion chromatograph (model No. 2110) with an AS 40 column. Plasma osmolality was determined by vapour pressure osmometry (Wescor model No. 5100B).

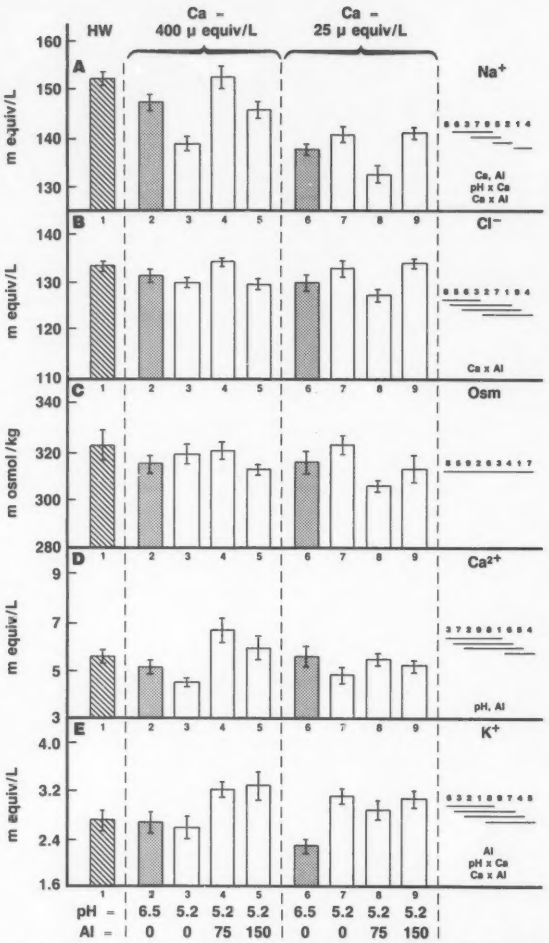


FIG. 1. Plasma (A) $Na^+$, (B) $Cl^-$, (C) total osmolality, (D) $Ca^{2+}$ and (E) $K^+$ in the blood of brook trout after 10 wk of exposure to eight different combinations of pH, $Ca^{2+}$, and Al in soft water, or to hard water (HW) at circumneutral pH (7.8) (cross-hatched). Values in the two exposures at circumneutral acidity (pH = 6.5) are stippled. Means ± 1 SEM for $N = 6$ in each cell. On the right are tabulated the variables which significantly affected ($p < 0.05$) the measured parameters overall, either individually or as interactive effects. Lines underscore subsets of means among which there were no significant differences; means not underscored with the same line were significantly different from one another ($p < 0.05$).

Net whole-body $Na^+$ flux rates ($J_{net}^{Na^+}$) were calculated by the Fick principle from the measured difference between inflow and outflow $Na^+$ levels and the flow rate through the box. Cumulative $Na^+$ losses over 48 h were estimated as the areas under curves relating $J_{net}^{Na^+}$ and time for individual fish. Mean cell Hb concentration (MCHC) was calculated as the ratio of Hb and Ht measurements for individual fish.

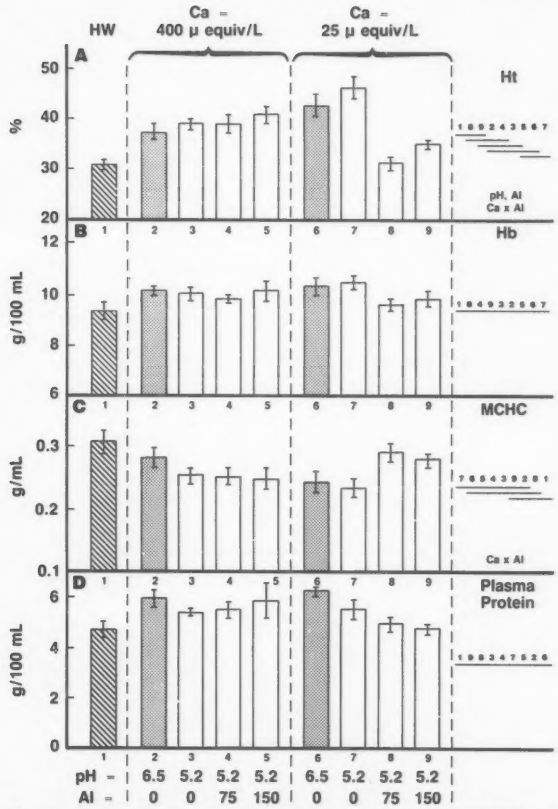


FIG. 2. (A) Ht, (B) Hb, (C) mean cell Hb concentration (MCHC), and (D) plasma protein concentration in the blood of brook trout after 10 wk of exposure to eight different combinations of pH, $Ca^{2+}$, and Al in soft water, or to hard water (HW) at circumneutral pH. Other details as in legend to Fig. 1.

### Treatment of Data

Data have been reported as means ± 1 SE ($N$) for each group (i.e. exposure condition). One-way analysis of variance was used to assess differences between groups; when the $F$ value indicated significance, Duncan's (1955) multiple range test was applied to discern specific differences. Two-way analysis of variance (pH × $Ca^{2+}$ at Al = 0; Al × $Ca^{2+}$ at pH = 5.2) was employed to detect specific pH, $Ca^{2+}$, Al, and interaction effects across the entire set of exposure conditions. Differences in $J_{net}^{Na+}$ from zero and changes in blood parameters within individual groups as a result of the 48-h challenges were evaluated by Student's two-tailed $t$-test, unpaired design. A 5% significance or protection level was used throughout.

## Results

### Exposure Conditions and Mortality

While agreement between intended and actual values of water chemistry was generally good, mean $Ca^{2+}$ at the higher concentration was about 15% below planned, and mean pH was up to 0.2 unit different from planned levels (Table 1). This partly reflected the resetting of intended acidic pH from 5.0 to 5.2 on day 16. The variation in $Na^+$ reflected cycling of the water-softening and deionizing systems. During the flux and challenge trials of this and the following paper (Wood et al. 1988b), the fluctuation was reduced, yielding a $Na^+$ level of 50–100 μequiv/L in different experiments, which varied less than 10% during any individual experiment. Speciation analysis revealed that almost all added Al was in the monomeric form in the tanks, but only about 25–33% of this was inorganic, due to complexation by organic wastes excreted by the fish. Mortality occurred only in the exposures at acidic pH with low $Ca^{2+}$ (Table 1) and ceased shortly after pH was reset to 5.2 on day 16. As this mortality was low, the effect of "selection" on the subsequent physiological data was minimal. Growth was depressed in these same treatments (see Mount et al. 1988).

### Effects of pH/$Ca^{2+}$/Al Exposures on Blood Composition

Ten weeks of exposure to a range of pH, $Ca^{2+}$, and Al caused significant variation in plasma $Na^+$ levels (Fig. 1A). The absolute differences were rather small, ranging from a high of 153 ± 2 mequiv/L ($N$ = 6) (at pH = 5.2, $Ca^{2+}$ = 400 μequiv/L, Al = 75 μg/L) to a low of 133 ± 2 mequiv/L in the same condition at low $Ca^{2+}$ (i.e. pH = 5.2, $Ca^{2+}$ = 25 μequiv/L, Al = 75 μg). While there was no direct pH effect, both elevated $Ca^{2+}$ and elevated Al acted alone to significantly raise plasma $Na^+$. These relationships were complicated by significant pH × $Ca^{2+}$ and $Ca^{2+}$ × Al interactions. In addition, plasma $Na^+$ was significantly higher in hard water than in either of the soft waters at circumneutral pH. Similar significant variation was seen in plasma $Cl^-$ levels, but to a much lesser extent (Fig. 1B). Overall there was a significant $Ca^{2+}$ × Al interaction.

Despite these differences in the two major ionic constituents of plasma, total osmolality did not vary significantly across exposure conditions, staying at a relatively uniform level of 305–325 mosmol/L (Fig. 1C). Interestingly, plasma protein, which contributes the colloidal fraction of total osmotic pressure, was similarly unaffected (Fig. 2D).

Plasma $Ca^{2+}$ levels varied significantly among exposure conditions in a pattern somewhat similar to that of $Na^+$ (compare Fig. 1D and 1A). On a relative basis, these differences were much greater (~50%) than those for $Na^+$ (~15%), plasma $Ca^{2+}$ ranging from a low of 4.5 ± 0.1 mequiv/L (at pH = 5.2, $Ca^{2+}$ = 400 μequiv/L, Al = 0 μg/L) to a high of 6.7 ± 0.5 mequiv/L (at pH = 5.2, $Ca^{2+}$ = 400 μequiv/L, Al = 75 μg/L). Overall, plasma $Ca^{2+}$ was significantly depressed by low pH and raised by elevated Al. Interestingly, water $Ca^{2+}$ itself did not exert a significant influence on plasma $Ca^{2+}$, and there were no hardwater versus softwater differences at circumneutral pH.

Plasma $K^+$ concentrations were also significantly affected by exposure condition, varying by ~50% from 2.3 ± 0.1 mequiv/L (at pH = 6.5, $Ca^{2+}$ = 25 μequiv/L) to 3.3 ± 0.2 μequiv/L (at pH = 5.2, $Ca^{2+}$ = 400 μequiv/L, Al = 150 μg/L) (Fig. 1E). The dominant influence was a stimulation by elevated Al; while there were no direct pH or $Ca^{2+}$ effects, significant pH × $Ca^{2+}$ and $Ca^{2+}$ × Al interactions were seen. Hardwater versus softwater differences at circumneutral pH were not significant.

Neither Hb (9.4–10.5 g/100 mL; Fig. 2B) nor plasma protein concentration (4.7–5.9 g/100 mL; Fig. 2D) was significantly affected by the exposure conditions. However, Ht varied considerably from a low of 31 ± 1% (in hardwater) to a high of 46 ± 3% (at pH = 5.2, $Ca^{2+}$ = 25 μequiv/L, Al = 0 μg/L) (Fig. 2A). Overall, Ht was significantly and separately raised by both low pH and elevated Al, and there was a $Ca^{2+}$ × Al

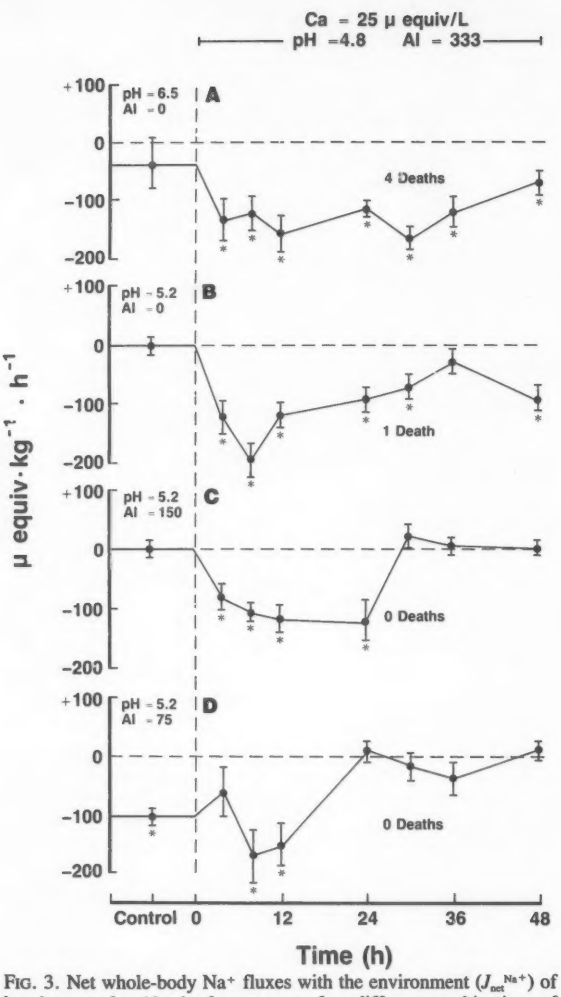


FIG. 3. Net whole-body $Na^+$ fluxes with the environment ($J_{net}^{Na^+}$) of brook trout after 10 wk of exposure to four different combinations of pH and Al at low $Ca^{2+}$ (25 μequiv/L) and during a 48-h challenge with pH = 4.8, $Ca^{2+}$ = 25 μequiv/L, Al = 333 μg/L. The exposure conditions were maintained during the control measurements. Means ± 1 SEM for $N = 6$ in each cell, except at 48 h where $N$ was reduced by the number of deaths tabulated. Asterisks indicate mean values of $J_{net}^{Na^+}$ which were significantly different from zero ($p < 0.05$).

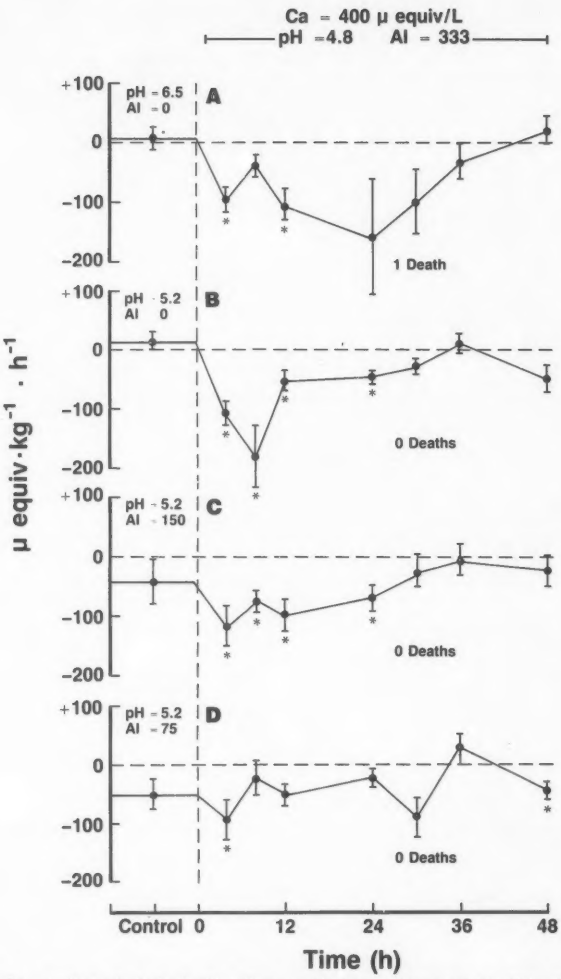


FIG. 4. Net whole-body $Na^+$ fluxes with the environment ($J_{net}^{Na^+}$) of brook trout after 10 wk of exposure to four different combinations of pH and Al at high $Ca^{2+}$ (400 μequiv/L) and during a 48-h challenge with pH = 4.8, $Ca^{2+}$ = 400 μequiv/L, Al = 333 μg/L. Other details as in legend to Fig. 3.

interaction. In addition, Ht was significantly higher in both of the soft waters at circumneutral pH than in hardwater. Calculated MCHC virtually mirrored these changes (compare Fig. 2C and 2A), although the overall effects were limited to a $Ca^{2+}$ × Al interaction.

### Effects of Exposure Conditions on $Na^+$ Fluxes and Mortality during Challenge

When tested in the water of their exposure condition (i.e. control measurements), all groups except one maintained a $J_{net}^{Na^+}$ which was not significantly different from zero (Fig. 3, 4), indicating that the fish were in whole-body $Na^+$ balance after 10 wk. The single exception was the group exposed to pH = 5.2, $Ca^{2+}$ = 25 μequiv/L, Al = 75 μg/L, where the net flux was significantly negative (Fig. 3D). However, as these same trout were later able to restore zero balance even in the presence of the acid/Al challenge, we are inclined to dismiss this initial imbalance as an experimental artifact, perhaps reflecting disturbance during the control measurements.

In the two groups held at pH = 6.5 (in the absence of Al), challenge with low pH = 4.8 alone resulted in a shift in $J_{net}^{Na^+}$ to moderately negative values, although the net flux became significantly different from zero at 0–8 h and 36–48 h only (data not shown). These changes were small relative to those seen with pH = 4.8 *plus* Al = 333 μg/L, indicating that the toxic effect of the latter challenge was mainly due to Al.

At the lower $Ca^{2+}$ level (25 μequiv/L), the acid plus Al challenge resulted in marked $Na^+$ losses which persisted throughout the 48-h period in fish previously naive to both Al and low pH (i.e. exposed to pH = 6.5, Al = 0 μg/L; Fig. 3A). Indeed, four of these six fish died during the final 12 h of the challenge. In the trout previously exposed to low pH alone (i.e. pH =

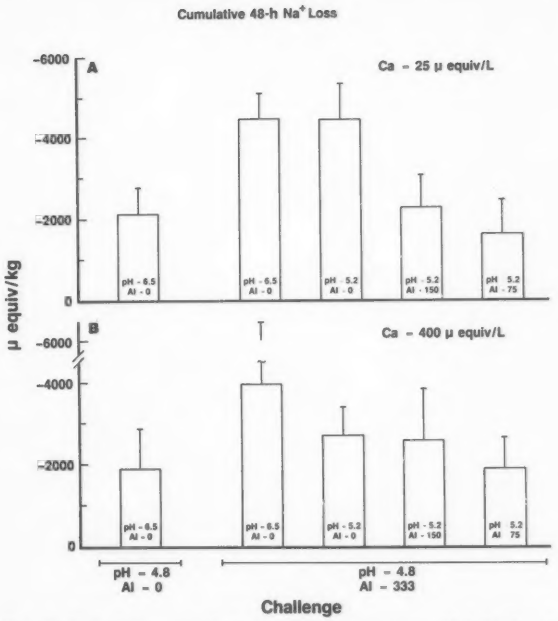


FIG. 5. Cumulative net whole-body $Na^+$ fluxes over 48 h for brook trout exposed to the low-pH or low-pH plus Al challenges shown. The condition of the previous 10 wk of exposure are indicated inside each bar. In treatments where fish died between 36 and 48 h (cf. Fig. 3, 4), the final measured $J_{net}^{Na^+}$ was used for these fish over the entire 12-h period. Means ± 1 SEM for $N = 6$ in each group.

5.2, Al = 0 μg/L), virtually identical $Na^+$ losses occurred during the challenge and one of the six fish died (Fig. 3B). However, prior exposure to low levels of Al (75 or 150 μg/L) in addition to low pH conferred increased resistance. Initial $Na^+$ loss rates during the challenge were similar to those in trout naive to Al, but zero net $Na^+$ balance was restored by 24 or 30 h (Fig. 3C, 3D). Furthermore, none of these fish died.

At the higher $Ca^{2+}$ level (400 μequiv/L), the overall trends were similar but less clearcut due to variability between animals, and the toxicity of the challenge appeared to be reduced (Fig. 4). Again, $Na^+$ loss rates during the challenge were least in the fish previously exposed to pH = 5.2 plus Al = 75 or 150 μg/L, and there were no mortalities in these groups.

When the data were analyzed in terms of cumulative whole-body $Na^+$ losses over 48 h for individual fish (Fig. 5), losses were greatest (~4000 μequiv/kg) in the groups naive to both low pH and Al and least (~1700 μequiv/kg) in the groups previously exposed to pH = 5.2, Al = 75 μg/L. There was no discernible effect of water $Ca^{2+}$ on cumulative $Na^+$ loss.

### Effects of Exposure Conditions on Blood Composition after Challenge

There were significant variations in some blood parameters as a result of the exposure conditions alone (Fig. 1, 2), so significant variations after the 48-h challenges would be expected regardless of challenge effects. The *changes* in blood composition resulting from the challenge are the more meaningful parameters and are presented in Fig. 6. At the lower $Ca^{2+}$ level (25 μequiv/L), plasma electrolyte changes were in general accord with the $Na^+$ flux data of Fig. 3 and 5A. Thus, a 48-h challenge with low pH = 4.8 alone had no effect on plasma $Na^+$ and $Cl^-$ concentrations in naive fish, but a challenge of the same group with pH = 4.8 plus Al = 333 μg/L caused highly significant decreases in both parameters (Fig. 6A). These effects of the acid/Al challenge were attenuated in fish previously exposed to pH = 5.2, Al = 0 μg/L and pH = 5.2, Al = 150 μg/L and abolished in the group exposed to pH = 5.2, Al = 75 μg/L. Decreases in plasma $Cl^-$ levels were generally greater than those of $Na^+$. Ht and plasma protein concentration fell after challenge of naive fish with low pH = 4.8 alone, but increased greatly when this same group was challenged with pH = 4.8 plus Al = 333 μg/L (Fig. 6C). In agreement with the $Na^+$ and $Cl^-$ changes, these hemoconcentration effects were attenuated by prior exposure to pH = 5.2, Al = 150 μg/L and abolished by prior exposure to pH = 5.2, Al = 75 μg/L. However, in disagreement with the electrolyte data, hemoconcentration was also prevented in the group previously held at pH = 5.2, Al = 0 μg/L.

At the higher $Ca^{2+}$ level (400 μequiv/L), effects of prior exposure on changes in blood parameters during the challenges were less clear, reflecting the same variability seen in the $Na^+$ flux data of Fig. 4 and 5B. Nevertheless, there were large decreases in plasma $Na^+$ and $Cl^-$ (Fig. 6B) and accompanying increases in Ht and plasma protein levels (Fig. 6D) in the naive fish under acid/Al challenge. Prior exposure to either low pH = 5.2 alone or pH = 5.2 plus 75 or 150 μg/L Al either reduced or abolished these effects.

## Discussion

### Water Chemistry

The present and following papers are the first physiological studies on fish chronically exposed to acid and Al for a prolonged period under defined softwater conditions. For acid stress alone, it is well documented that the water quality parameter of key importance in modifying both toxicological and physiological responses is $Ca^{2+}$ (e.g. Brown 1981, 1983; Wood 1988), which was controlled closely in the present experiments (Table 1). The fluctuations in water $Na^+$ during the 10 wk of exposure probably had minimal influence on the overall results because they occurred simultaneously in all exposure tanks as the water treatment system cycled. Furthermore, Brown (1981) found little effect of water $Na^+$ over the range of variation noted here on the toxicity of acid to brown trout (*Salmo trutta*). During the challenge experiments, the variation was greatly reduced to levels representative of very dilute natural soft water (50–100 μequiv/L). Based on branchial $Na^+$ transport studies in these fish (McDonald and Milligan 1988), the influence of this small variation on the present results would have been negligible. Total Al levels were well controlled during both exposure and challenge experiments, although the contribution of the inorganic monomeric fraction was rather variable, probably due to variable complexation by organics originating from food and excreta in the water. Al speciation was not measured during the challenges, but the faster water turnover and the lack of feeding here probably resulted in a greater inorganic monomeric fraction, which would tend to increase toxicity.

### Effects of pH/$Ca^{2+}$/Al Exposures on Ionoregulatory Parameters

Ten weeks of exposure to a range of sublethal pH/$Ca^{2+}$/Al conditions induced significant variation in plasma electrolyte

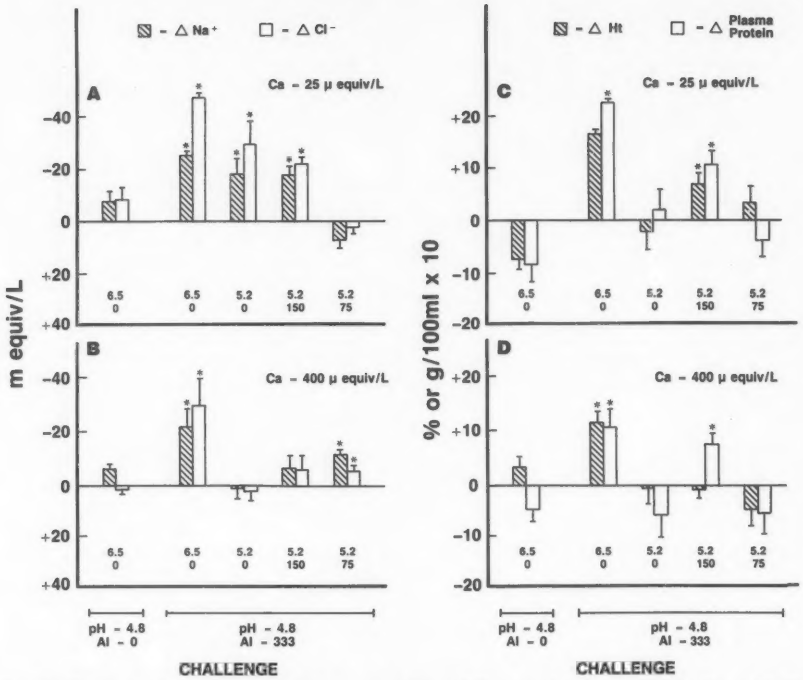


FIG. 6. Changes, relative to the respective mean values of Fig. 1 and 2, in plasma (A) $Na^+$, (B) $Cl^-$, (C) Ht, and (D) protein levels of surviving brook trout after 48-h challenges with low pH, or low pH plus Al as shown. The conditions of the previous 10 wk of exposure are indicated below each set of bars. Means ± 1 SEM for $N = 6$ in each cell, or where deaths occurred, for an $N$ identical to that at 48 h for that treatment in Fig. 3 and 4. Asterisks indicate means for which the changes were significant ($p < 0.05$).

concentrations (Fig. 1). For $Na^+$ and $Cl^-$, the absolute sizes of these chronic effects were rather small. Indeed, the total variation among the different exposure conditions was within the normal range which we (Booth et al. 1988) and other workers (e.g. Houston et al. 1971; McCormick and Naiman 1984) have observed for brook trout maintained at circumneutral pH in either hard or soft water. Furthermore, there was no significant variation in plasma osmolality (Fig. 1C), of which $Na^+$ and $Cl^-$ are the major constituents. This is possibly explained by the mobilization of other osmolytes such as glucose and taurine during long-term exposures (Fugelli and Vislie 1982; Giles et al. 1984; Scherer et al. 1986).

Qualitatively, *chronic* effects on $Na^+$ and $Cl^-$ regulation were very different from *acute* effects. Thus after 10 wk, there was no significant overall influence of low pH, elevated Al had a significant *positive* influence on plasma $Na^+$ (Fig. 1A), and the fish were in net $Na^+$ balance in most conditions (Fig. 3, 4). The acute effect of all the acid and acid/Al conditions (i.e. pH = 5.2, Al = 0–150 μg/L) would have been an initial loss of $Na^+$ and $Cl^-$ across the gills and depression of plasma levels, followed by a partial recovery over the next few days due mainly to reduction of the unidirectional efflux components (Booth et al. 1988). Taken together, these observations suggest that the recovery process continues during chronic exposure, resulting in adaptation to the sublethal stresses.

There have been a number of previous studies on the chronic ionoregulatory effects (2 wk to 3 mo) of low pH exposure alone (4.5–6.0) on other salmonids (rainbow trout, brown trout, Atlantic salmon (*Salmo salar*)), each of which is more acid sensitive than brook trout (Grande et al. 1978). Recovery of plasma $Na^+$, $Ca^-$, and osmolality was at best partial in all investigations (McWilliams 1980; Lee et al. 1983; Saunders et al. 1983; Johnston et al. 1984; Giles et al. 1984; Lacroix et al. 1985; Weiner et al. 1986; Brown et al. 1986). Thus the present study with brook trout is the first to show complete recovery of these parameters, at least at pH = 5.2. However, in the same species at a more severe pH (≃4.6), Leivestad et al. (1976) found that plasma $Na^+$ and $Cl^-$ were still depressed after 1 yr of exposure, suggesting that complete recovery may be blocked by a threshold somewhere between 5.2 and 4.6.

The significant depression of $Ca^{2+}$ levels caused by chronic exposure to low pH alone in the present study (Fig. 1D) has been observed previously in other salmonids (Wood and McDonald 1982; Scherer et al. 1986; Weiner et al. 1986). The mechanism is unknown. The depressions occur despite the minimal effects of acute acid exposure on $Ca^{2+}$ exchange with the environment (Hõbe et al. 1984; Booth et al. 1988) and the lack of chronic effects of low pH on whole-body $Ca^{2+}$ dynamics in brook trout (Rodgers 1984). As impairment of $Ca^{2+}$ regulation has been implicated in reproductive failure during long-term acid stress, this is clearly an important area for further investigation.

The present study is the first to examine the ionoregulatory effects of long-term acid plus Al exposure. The results, which show a significant supportive effect of Al on plasma $Na^+$, $Ca^{2+}$, and $K^+$ levels (Fig. 1), are unexpected in view of the normally

toxic action of Al on ionoregulation. They suggest that low levels of Al may actually be beneficial during chronic acid exposure. At present, we can only speculate that this reflects some sort of recovery "overshoot," a view supported by subsequent studies on $Na^+$ uptake kinetics in these same fish (McDonald and Milligan 1988). Alternatively or additionally, the small fraction of Al present as $Al^{3+}$ at pH = 5.2 may have exerted a $Ca^{2+}$-like action in stabilizing branchial permeability. Schofield and Trojnar (1980) and Baker and Schofield (1982) have suggested that this action is the basis of the protective effect of Al seen at somewhat lower pH's.

There was no chronic influence of any of the pH/$Ca^{2+}$/Al exposures on either blood Hb or plasma protein levels (Fig. 2B, 2D). Thus, $O_2$ and $CO_2$ transport capacity, blood buffering capacity, and plasma oncotic pressure were probably little affected. Nevertheless, there were substantial variations in Ht due to variations in MCHC (Fig. 2A, 2C). The two most likely explanations are erythrocytic swelling and/or a greater fraction of Hb-poor erythrocytes (e.g. reticulocytes) in the circulation under chronic sublethal stress. Both effects are seen during acute lethal acid stress (Milligan and Wood 1982). The more dilute intracellular environment of swollen erythrocytes will improve the $O_2$ affinity of the blood (Nikinmaa 1986).

### Effects of Exposure Conditions on Responses to Challenge

In terms of both mortality and net $Na^+$ loss rates, Wyoming trout naive to acid and Al responded similarly to the naive Ontario trout of our companion studies (Booth et al. 1988), although the challenge protocols were slightly different. As before, all the mortality and the major part of the $Na^+$ losses were clearly attributable to the Al component of the exposure at pH = 4.8, Al = 333 μg/L. The resultant reductions in plasma ions and increases in Ht and plasma protein levels due to internal fluid shifts were also similar to those seen in the Ontario trout (Booth et al. 1988; Wood et al. 1988a). Thus there did not appear to be significant differences in sensitivity associated with the differences in fish stocks, water quality, and altitude.

We have characterized the branchial ion loss response of naive brook trout to acid/Al challenge as comprising two phases (Booth et al. 1988). Over the first 6–12 h, there is an initial "shock" phase of high salt loss due to both an inhibition of active uptake and a more important stimulation of diffusive efflux. By 24 h, this is replaced by a phase of partial "recovery" in which the diffusive efflux is returned to control levels or lower, while the inhibition of active uptake largely persists.

In the present study, there was no detectable effect of the preexposure condition on $Na^+$ loss rates during the initial shock phase (Fig. 4, 5). There was, however, a clear acceleration of the recovery phase in trout previously exposed to lower levels of Al (75 or 150 μg/L) at pH = 5.2, $Ca^{2+}$ = 25 μequiv/L (Fig. 3C, 3D). Thus by 24–30 h, the animals had returned to zero $Na^+$ balance, resulting in a halving of the total $Na^+$ loss over 48 h (Fig. 5A) and a prevention of mortality during this period. Plasma $Na^+$ and $Cl^-$ depressions and hematological changes were similarly attenuated (Fig. 6A, 6C). Prior exposure to Al = 75 μg/L appeared to be slightly more effective than to 150 μg/L in conferring this increased resistance. At the higher $Ca^{2+}$ = 400 μequiv/L, the effects were less clear due to variability among fish. Nevertheless the overall pattern as shown by net $Na^+$ flux rates (Fig. 4), cumulative losses (Fig. 5), and plasma changes (Fig. 6B, 6D) was similar to that at low $Ca^{2+}$. In total, these results indicate that chronic exposure to low levels of Al and acidity induced physiological acclimation in the traditional sense — increased resistance of a more severe exposure.

The results with prior exposure to low pH = 5.2 alone were equivocal. Mortality during acid/Al was reduced but not prevented, $Na^+$ loss rates remained high (Fig. 3B, 4B, 5), yet plasma $Na^+$ and $Cl^-$ and hematological changes were reduced or abolished (Fig. 6). The protective effect seemed greater at the higher $Ca^{2+}$. These results are perhaps to be expected inasmuch as most of the toxic effect of the challenge was due to the Al component, to which these fish were still naive. Any increased resistance could be explained by the fact that a major action of Al is similar to that of acid alone (ionoregulatory impairment) and that morphological changes in the gills are somewhat similar during chronic sublethal acid exposure as during acid plus Al exposure (see below) (Tietge et al. 1988). However, our more detailed physiological studies indicate that prior exposure to acid alone does not provide significant acclimatory resistance to acid plus Al stress (Wood et al. 1988b; McDonald and Milligan 1988).

Several studies have described dramatic morphological changes in the gills associated with chronic exposure to sublethal acid/Al stress (Chevalier et al. 1985; Karlsson-Norrgren et al. 1986a; 1986b; Tietge et al. 1988). These include deformation and fusion of lamellae, epithelial detachment, a general swelling of the lamellae due to epithelial hyperplasia, white blood cell infiltration of the lymphatic spaces, increased prevalence of "dense" cells, and most notably a gross proliferation of chloride cells, many of which may contain Al inclusion bodies. Blood to water diffusion distance clearly increases. Based on these descriptions, one might speculate that the acclimatory resistance to acid/Al challenge results from the increased diffusion distance limiting passive ion efflux, while the greater number of chloride cells limits the inhibition of active uptake. However, this scenario predicts that $Na^+$ losses should have been reduced primarily during the initial shock phase and not as observed during the later recovery phase. Perhaps in Al-acclimated fish, the recovery phase is different, involving an activation of the greater uptake capacity of the proliferated chloride cells (cf. McDonald and Milligan 1988) or a more effective reduction of the passive permeability.

In summary, the present results indicate that brook trout adapt to chronic sublethal acid/Al stress. At least at the Al levels employed here, the fish demonstrate acclimation rather than sensitization, in accord with the results of Orr et al. (1986) on rainbow trout. The acclimation has a definite physiological basis reflected in ionoregulatory parameters. We suggest that this acclimation process allows fish to survive in the wild under conditions where they might otherwise succumb. Specificially, fish chronically exposed to low levels of Al in mildly acidic soft water may possess increased resistance to short-term increases in Al and acidity associated with episodic "surges."

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# Physiological Evidence of Acclimation to Acid/Aluminum Stress in Adult Brook Trout (*Salvelinus fontinalis*). 2. Blood Parameters by Cannulation

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Wood, C. M., B. P. Simons, D. R. Mount, and H. L. Bergman. 1988. Physiological evidence of acclimation to acid/aluminum stress in adult brook trout (*Salvelinus fontinalis*). 2. Blood parameters by cannulation. Can. J. Fish. Aquat. Sci. 45: 1597–1605.

Brook trout (*Salvelinus fontinalis*) exposed for 10 wk to sublethal acid (pH = 5.2) plus Al (150 μg/L) in flowing soft water ($Ca^{2+}$ = 25 μequiv/L) did not exhibit chronic respiratory disturbance or elevated stress indices, as revealed by sampling of arterial blood gases, acid–base status, glucose, and cortisol via an indwelling catheter. Acclimation occurred, which prevented mortality and greatly attenuated the disturbances of respiratory, acid–base, and stress parameters normally seen upon challenge with more severe acid (pH = 4.8) plus Al conditions (333 μg/L) for 3 d. Ionoregulatory, fluid volume, and hematological disturbances were similarly reduced. Higher water $Ca^{2+}$ (400 μequiv/L) slightly delayed but did not prevent this suite of toxic responses in naive fish. These disturbances did not occur in naive fish challenged with acid alone (pH = 4.8). However, long-term adaptation to acid alone (pH = 5.2) resulted in elevated glucose and cortisol levels and offered no protection against the more severe acid plus Al challenge. Thus the acclimation was to Al rather than to acidity itself, and low levels of Al may be beneficial to fish under chronic acid stress.

Des ombles de fontaine (*Salvelinus fontinalis*) exposés pendant 10 sem à des concentrations sublétales d'acide (pH = 5,2) et à de l'Al (150 μg/L) en eau douce à débit continu ($Ca^{+2}$ = 25 μequiv/L) n'ont pas montré de perturbations respiratoires chroniques ou un stress élevé après échantillonnage à l'aide d'une sonde à demeure des gaz sanguins artériels, de l'équilibre acidobasique, du glucose et du cortisol. On a observé une accoutumance qui a éliminé la mortalité et a grandement atténué les perturbations des paramètres respiratoires, de l'équilibre acidobasique et du stress, normalement observées après provocation dans un milieu à teneurs en acide (pH = 4,8) et en Al (333 μg/L) plus élevées pendant 3 d. De même, les perturbations ionorégulatoires, hématologiques et volumétriques des fluides ont été réduites. Une teneur aqueuse en $Ca^{+2}$ plus élevée (400 μequiv/L) a légèrement retardé cette séquence de réactions toxiques chez les poissons naïfs mais ne l'a pas éliminée. Ces perturbations n'ont pas été observées chez les poissons naïfs provoqués dans un milieu acide seulement (pH = 4,8). Toutefois, une adaptation à long terme à un milieu acide seulement (pH = 5,2) a entraîné des niveaux élevés de glucose et de cortisol et n'a pas offert de protection contre la provocation plus violente à l'acide ajouté d'Al. Ainsi, l'accoutumance ne concerne que l'Al et non l'acidité même et de faibles teneurs en Al peuvent être bénéfiques pour les poissons faisant l'objet d'un stress à l'acide chronique.

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In the preceding study, we demonstrated that brook trout (*Salvelinus fontinalis*) exposed for 10 wk to a range of sublethal pH, $Ca^{2+}$, and Al conditions show adaptation as manifested by regulation of plasma ions and hematology (Wood et al. 1988a). However, stress indices such as blood glucose or cortisol (Donaldson 1981; Wedemeyer and McLeay 1981) were not measured, so it was not possible to conclude whether sublethal stress persisted despite adaptation of ionoregulatory function. Respiratory and acid–base parameters were also not assessed. Morphological observations on the gills of trout exposed for extended periods to sublethal acid/Al conditions have shown a general thickening of the lamellar epithelium and associated increase in the water to blood diffusion distance (Chevalier et al. 1985; Tietge et al. 1988; Karlsson-Norrgren et al. 1986a, 1986b). Such changes might well cause a chronic limitation of respiratory gas exchange and associated disturbance of acid–base balance, thereby contributing to sublethal stress. Therefore the first objective of the present study was to determine whether chronic respiratory limitation and/or sublethal stress occurred in these fish after 10 wk of exposure. Cannulation was employed for blood sampling (cf. Wood et al. 1988b), permitting repetitive determination of blood gases, acid–base status, glucose, cortisol, and other parameters with minimal disturbance to the fish.

The preceding study also demonstrated that 10 wk of exposure to sublethal levels of Al and acid together resulted in acc-

limation, as evidenced by greater survival and smaller ionoregulatory disturbances during a more severe acid plus Al challenge (Wood et al. 1988a). However, respiratory disturbance and acidosis are also important components of the lethal response to acid/Al (Rosseland 1980; Neville 1985; Wood et al. 1988b). We anticipated that such disturbances might well be worsened in trout already burdened with thickened lamellar epithelia as a result of long-term sublethal exposure (Tietge et al. 1988). Therefore the second objective of the present study was to employ chronic cannulation to assess whether respiratory, acid–base, and stress responses to a more severe challenge were exacerbated as a result of long-term sublethal exposure, or rather were attenuated by acclimation. By permitting repetitive blood sampling, these experiments allowed a more detailed examination of the time course of blood responses to challenge in the various exposure groups. They therefore clarified the role of water $Ca^{2+}$ and answered the question whether prior exposure to sublethal acidity alone also provided acclimatory resistance to combined acid/Al challenge, for the results of the preceding study (Wood et al. 1988a) were equivocal on this point.

## Materials and Methods

### Experimental Animals, Exposures, and Challenge Conditions

Adult brook trout were exposed for 10 wk to different combinations of pH, $Ca^{2+}$, and Al in flowing artificial soft water at the Fish Physiology and Toxicology Laboratory, Laramie, Wyoming (altitude = 2200 m). Details on fish origin, holding, and water chemistry have been described in the preceding paper (Wood et al. 1988a). The overall experimental plan was first to examine blood parameters (by arterial cannulation) while the fish remained in their exposure water in order to detect whether chronic respiratory effects and/or sublethal stress occurred after 10 wk of exposure. These same trout were then challenged with a higher Al/lower pH condition (potentially lethal) for 3 d. During this period, blood was repetitively sampled via the catheter to assess the nature and time course of respiratory and other disturbances and the extent of acclimatory resistance offered by the prior exposure conditions.

Fish were exposed for 10 wk to four different combinations of pH, $Ca^{2+}$, and Al (Table 1). These combinations were chosen to assess the effects of low $Ca^{2+}$ (25 μequiv/L) versus higher $Ca^{2+}$ (400 μequiv/L) within the softwater range, the effects of exposure to low pH (5.2) alone, and the effects of combined exposure to low pH (5.2) plus Al (150 μg/L). Fish from each exposure were then subjected to the more severe combined acid (pH = 4.8) plus Al (333 μg/L) challenge. In addition, fish from the pH = 6.5, $Ca^{2+}$ = 25 μequiv/L, Al = 0 μg/L exposure were challenged with low pH (4.8) alone to discern the effects of acidity by itself.

Arterial catheters were implanted via the caudal artery as described previously (Wood et al. 1988b). The preexposure water was used for anaesthesia (50 mg MS-222/L, Sigma) on the operating table, and the pH was held at the appropriate level by addition of KOH. After cannulation, the fish were transferred to individual flux boxes served with a continuous flow (~0.5 $L \cdot kg^{-1} \cdot min^{-1}$) of water *identical in composition to that of their exposure condition* (cf. Wood et al. 1988a). The $P_{O_2}$ of the inflowing water was maintained above 115 torr, $P_{CO_2}$ below 1 Torr and temperature at 11 ± 1°C.

After 48 h of recovery, a blood sample was taken from each fish while it remained in its exposure water to determine chronic effects on blood parameters. The measurements from this sample also served as the *control* values for the subsequent challenges. The water was then changed to the appropriate challenge condition (Table 1) and samples taken at 4, 18, 28, 42, and 66 h, if death did not occur earlier. Observations for mortalities were continued until 72 h.

All blood samples (700 μL) were withdrawn anaerobically from the arterial catheters into gas-tight, ice-cold Hamilton syringes and immediately replaced by reinfusion of an equal volume of nonheparinized Cortland saline (Wolf 1963). Samples were analyzed for arterial $O_2$ tension ($Pa_{O_2}$), pH (pHa), red blood cell intracellular pH (RBC pHi), total $O_2$ in whole blood ($Ca_{O_2}$), total $CO_2$ in whole blood ($Ca_{CO_2}$) and true plasma, hematocrit (Ht), hemoglobin (Hb), lactate, and glucose, and plasma levels of $Na^+$, $Cl^-$, $K^+$, $Ca^{2+}$, total protein, and cortisol.

### Analytical Techniques and Calculations

Analytical techniques were identical to those described by Wood et al. (1988b), but with the following additional measurements. Plasma cortisol was determined on 20-μL samples using a Corning $^{125}I$ radioimmunoassay kit. RBC pHi was measured by the freeze-thaw red cell lysate method of Zeidler and Kim (1977), as validated for trout cells by Milligan and Wood (1985). The same Radiometer pH microelectrode (type E5021) was employed for the lysate as for the whole-blood pHa measurement, and the red cell pellet was obtained from the sample centrifuged at 9000 × *g* for 2 min to separate plasma for ions and cortisol. $Ca_{O_2}$ was determined by means of a Lex-$O_2$-Con analyzer (Lexington Instruments), using a sample volume of

TABLE 1. Intended exposure and challenge conditions for each experimental group, and mortalities and median survival times during each 72-h challenge.

| Exposure | | | Challenge | | | | |
|---|---|---|---|---|---|---|---|
| pH | $Ca^{2+}$ (μequiv/L) | Al (μg/L) | pH | $Ca^{2+}$ (μequiv/L) | Al (μg/L) | Mortality (during 72-h challenge) | LT50 (h) (with 95% CL) |
| 6.5 | 25 | 0 | 4.8 | 25 | 0 | 0/6 | >72 |
| 6.5 | 25 | 0 | 4.8 | 25 | 333 | 6/6 | 34.1 (19.9 – 52.0) |
| 6.5 | 400 | 0 | 4.8 | 400 | 333 | 5/6 | 53.0 (38.1 – 73.7) |
| 5.2 | 25 | 0 | 4.8 | 25 | 333 | 6/7 | 31.6 (21.2 – 54.5) |
| 5.2 | 25 | 150 | 4.8 | 25 | 333 | 0/6 | >72 |

50 μL and the recalibration procedure described by Wood et al. (1979).

The following parameters were calculated as described by Wood et al. (1988b): $Pa_{CO_2}$, plasma bicarbonate concentration ($HCO_3^-$), mean cell Hb concentration (MCHC), and the concentration of metabolic $H^+$ added to the whole blood ($\Delta H^+m$). As $Ca_{O_2}$ was measured directly in the present study, Hb-bound $O_2$ per unit Hb ($[O_2]/[Hb]$) was calculated as

$$(1) \quad [O_2]/[Hb] = \frac{Ca_{O_2} - (Pa_{O_2} \cdot \alpha O_2)}{[Hb]}$$

where $\alpha O_2$ represents the tabulated value (Boutilier et al. 1984) for $O_2$ solubility in trout plasma at the experimental temperature. The inverse of the ratio of the final measured plasma protein concentration to the initial value in each fish was employed as an index of the change in plasma volume, as described by McDonald et al. (1980).

### Treatment of Data

Median survival times (LT50) and their 95% confidence limits were calculated by standard log/probit analysis and nomographic methods (Litchfield 1949; Litchfield and Wilcoxin 1949). Other data have been generally expressed as means ± 1 SE (*N*) for each group. Comparisons amongst values taken when the fish were still under their four different exposure conditions, or amongst final values after challenge, were performed by one-way analysis of variance, followed by Duncan's (1955) multiple range test when the *F* value indicated significance.

As discussed previously (Wood et al. 1988b), it is difficult to accurately portray mean responses for physiological data when members of the group die at different times as a consequence of the experimental treatment. This was a problem for three of the challenge treatments in the present study where mortality was 6/6, 5/6, and 6/7, but not for two others where survival was 100% over 72 h. Therefore, the approach adopted was to show a few key parameters as plots of individual data in groups exhibiting mortality and as means ± 1 SEM in groups exhibiting 100% survival. For all other parameters, data were summarized as means ± 1 SEM for final values, where the final value represented either the 66-h measurement or the last measurement prior to death. In the three groups exhibiting mortality, even one of the two survivors appeared close to death at 72 h as evidenced by laboured breathing, poor colouration, and disturbed physiological parameters. Thus for these groups, the final means were representative of the blood status shortly prior to death and therefore comparable with the "terminal" data of Wood et al. (1988b). In all groups, there was a paired design, so Student's two-tailed paired *t*-test ($p \leq 0.05$) was used for comparisons between the control values taken under the exposure condition and the intervening or final values taken under the challenge condition.

## Results

### Chronic Respiratory and Stress Effects of pH/$Ca^{2+}$/Al Exposures

When fish were examined in the conditions to which they had been exposed for 10 wk, there were no significant differences in $Pa_{O_2}$, $[O_2]/[Hb]$, or $Pa_{CO_2}$, suggesting that neither sublethal acid alone nor acid plus Al caused chronic respiratory problems, at least under resting conditions (Table 2). Indeed, blood lactate was significantly lower in fish exposed to sublethal acid plus Al than in those held at pH = 6.5, $Ca^{2+}$ = 25 μequiv/L, Al = 0 μg/L. There were significant variations in blood acid–base status, but these resulted from

TABLE 2. Respiratory, acid–base, and stress parameters in the arterial blood of brook trout after 10 wk of exposure to various pH/$Ca^{2+}$/Al conditions. Means ± 1 SEM.

| | Exposure conditions | | | |
|---|---|---|---|---|
| | pH = 6.5<br>$Ca^{2+}$ = 25 μequiv/L<br>Al = 0 μg/L<br>*N* = 12 | pH = 6.5<br>$Ca^{2+}$ = 400 μequiv/L<br>Al = 0 μg/L<br>*N* = 6 | pH = 5.2<br>$Ca^{2+}$ = 25 μequiv/L<br>Al = 0 μg/L<br>*N* = 7 | pH = 5.2<br>$Ca^{2+}$ = 25 μequiv/L<br>Al = 150 μg/L<br>*N* = 6 |
| $Pa_{O_2}$ (Torr) | 65.6 ± 3.0 | 76.4 ± 3.9 | 62.8 ± 3.8 | 68.7 ± 6.0 |
| $[O_2]/[Hb]$ (μmol/g) | 58.2 ± 1.2 | 56.7 ± 2.0 | 63.0 ± 1.3 | 61.0 ± 1.1 |
| $Pa_{CO_2}$ (Torr) | 2.28 ± 0.12 | 2.63 ± 0.19 | 1.99 ± 0.24 | 2.83 ± 0.24 |
| $[HCO_3^-]$ (mequiv/L) | 9.74 ± 0.88 | 6.84 ± 0.56[a] | 8.34 ± 1.11 | 7.82 ± 0.65 |
| pHa | 7.957 ± 0.036 | 7.769 ± 0.025[a] | 7.914 ± 0.084 | 7.795 ± 0.048 |
| RBC pHi | 7.405 ± 0.025 | 7.375 ± 0.018 | 7.437 ± 0.038 | 7.367 ± 0.022 |
| MCHC (g/mL) | 0.294 ± 0.006 | 0.324 ± 0.013 | 0.279 ± 0.009 | 0.291 ± 0.021 |
| Lactate (mequiv/L) | 1.07 ± 0.10 | 0.78 ± 0.06 | 0.78 ± 0.13 | 0.59 ± 0.15[a] |
| Glucose (mmol/L) | 3.98 ± 0.36 | 4.47 ± 0.35 | 5.83 ± 0.67[a] | 5.07 ± 0.44 |
| Cortisol (ng/mL) | 156.3 ± 19.7 | 162.3 ± 34.9 | 312.5 ± 44.7[a] | 148.8 ± 23.4[b] |

[a]Significantly different ($p < 0.05$) from comparable value at pH = 6.5, $Ca^{2+}$ = 25 μequiv/L, Al = 0 μg/L.
[b]Significantly different ($p < 0.05$) from comparable value at pH = 5.2, $Ca^{2+}$ = 25 μequiv/L, Al = 0 μg/L.

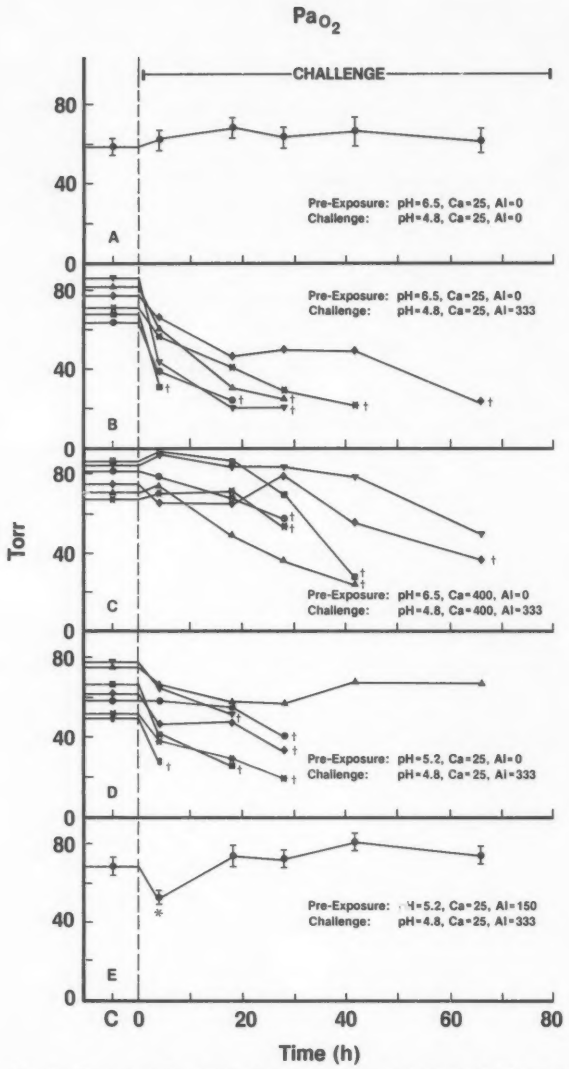


FIG. 1. Changes in $Pa_{O_2}$ in brook trout challenged for 3 d with (A) acid alone (pH = 4.8) or (B–E) acid plus Al (pH = 4.8, Al = 333 μg/L) in flowing artificial soft water. The fish had been previously exposed to various pH/$Ca^{2+}$/Al conditions for 10 wk ($Ca^{2+}$ = μequiv/L; Al = μg/L). Data are means ± 1 SEM ($N$ = 6) in groups with 100% survival until 72 h and are for individual fish ($N$ = 6–7) in groups with 83–100% mortality until 72 h († signifies death). A different symbol is used for each fish. Asterisks indicate means significantly different ($p < 0.05$) from the control mean taken from these same fish in their exposure water prior to challenge.

differences in water $Ca^{2+}$ levels rather than from sublethal acid or Al exposure (Table 2). Thus, plasma $HCO_3^-$ and pHa were significantly lower at the higher water $Ca^{2+}$ level. RBC pHi and MCHC remained constant in the face of this variation (Table 2). Hb, plasma protein, and ions ($Na^+$, $Cl^-$, $Ca^{2+}$, and $K^+$) were also unaffected by exposure to acid alone or acid plus Al (data not shown). However, $Na^+$, $Cl^-$, and $Ca^{2+}$ levels were all significantly elevated at the higher water $Ca^{2+}$ concentration.

Despite the absence of respiratory and ionoregulatory effects, significant elevations in both blood glucose and cortisol indicated a chronic stress response in the fish exposed to pH = 5.2, $Ca^{2+}$ = 25 μequiv/L, Al = 0 μg/L (Table 2). Interestingly, this was not seen at the same pH in the presence of 150 μg Al/L, suggesting that under these circumstances, Al may have exerted a protective action.

## Effects of Exposure Conditions on Mortality during Challenge

The survival data alone clearly indicated the toxicity of the challenges to the various groups (Table 1). Fish that were naive to both acid and Al did not die when challenged with acid alone (pH = 4.8 at $Ca^{2+}$ = 25 μequiv/L) for 72 h, but fish from the same exposure all died when challenged with acid *plus* Al (333 μg/L). Exposure and acid/Al challenge at the higher $Ca^{2+}$ = 400 μequiv/L did not appreciably affect mortality and only slightly extended LT50. The one survivor at 72 h appeared to be close to death. Prior exposure to sublethal acid alone also had no ameliorative effect on mortality or LT50 during the acid/Al challenge, although the sole survivor at 72 h appeared to be reasonably healthy. However, prior exposure to sublethal acid plus Al (pH = 5.2, $Ca^{2+}$ = 25 μequiv/L, Al = 150 μg/L) had a dramatic protective effect, for there was no mortality during the 72-h challenge with pH = 4.8, $Ca^{2+}$ = 25 μequiv/L, Al = 333 μg/L (Table 1).

Relative to noncannulated Wyoming fish subjected to identical exposure regimes and 48-h challenges, these mortality rates were comparable or slightly greater (Wood et al. 1988a). Relative to cannulated Ontario fish tested in comparable challenges (Wood et al. 1988b), these rates were essentially identical.

## Effects of Exposure Conditions on Respiratory and Acid–Base Responses to Challenge

The $Pa_{O_2}$ data were representative of respiratory and acid–base responses in general and therefore have been shown in detail in Fig. 1. In fish naive to both acid and Al at $Ca^{2+}$ = 25 μequiv/L, challenge with acid alone (pH = 4.8) had no effect on $Pa_{O_2}$ (Fig. 1A), but challenge with acid plus Al (pH = 4.8, Al = 333 μg/L) caused a rapid and dramatic fall in $Pa_{O_2}$ to values typical of venous blood prior to death (Fig. 1B). Prior exposure and challenge at the higher $Ca^{2+}$ level only slightly delayed this effect (Fig. 1C), while prior exposure to acid alone (pH = 5.2) offered no protective influence at all (Fig. 1D). In contrast, fish previously exposed to acid plus Al showed a transitory fall in $PaO_2$ after 4 h, followed by a complete return to resting values for the remainder of the challenge period (Fig. 1E).

Very similar trends were seen for other respiratory parameters (Table 3). Thus, final values of $[O_2]$/[Hb] were significantly depressed in all groups challenged with acid plus Al, except for the group previously exposed to pH = 5.2, Al = 150 μg/L. $Pa_{CO_2}$ increased in mirror image to the decreases in $Pa_{O_2}$ during challenge, again with the exception of the trout previously exposed to acid plus Al. Here, $Pa_{CO_2}$ actually decreased below resting levels by the final sample of the challenge period. Challenge of naive fish with acid alone exerted no significant respiratory or acid–base effects (data not shown).

If decreases in arterial blood oxygenation are severe enough to impair $O_2$ delivery to the tissues, then the production of lactic acid by anaerobic metabolism may occur. Blood lactate meas-

TABLE 3. Final challenge parameters in the arterial blood of brook trout exposed to various pH/$Ca^{2+}$/Al conditions for 10 wk. The fish were challenged with pH = 4.8, Al = 333 μg/L at the exposure $Ca^{2+}$ level; the tabulated final values represent either the 66-h measurements (for survivors) or the last measurement prior to death (for mortalities).

| | Exposure conditions | | | |
|---|---|---|---|---|
| | pH = 6.5<br>$Ca^{2+}$ = 25 μequiv/L<br>Al = 0 μg/L<br>*N* = 6 | pH = 6.5<br>$Ca^{2+}$ = 400 μequiv/L<br>Al = 0 μg/L<br>*N* = 6 | pH = 5.2<br>$Ca^{2+}$ = 25 μequiv/L<br>Al = 0 μg/L<br>*N* = 7 | pH = 5.2<br>$Ca^{2+}$ = 25 μequiv/L<br>Al = 150 μg/L<br>*N* = 6 |
| $[O_2]/[Hb]$ (μmol/g) | 45.0 ± 5.1[a] | 41.2 ± 5.6[a] | 50.8 ± 4.4[a] | 59.3 ± 1.2[b] |
| $Pa_{CO_2}$ (Torr) | 3.87 ± 0.40[a] | 2.94 ± 0.36 | 3.12 ± 0.40[a] | 2.34 ± 0.21[a,b] |
| $[HCO_3^-]$ (mequiv/L) | 8.96 ± 1.10 | 4.74 ± 0.71[a,b] | 7.60 ± 0.92 | 5.98 ± 0.47[a,b] |
| pHa | 7.715 ± 0.008[a] | 7.567 ± 0.046[a,b] | 7.742 ± 0.045[a] | 7.762 ± 0.015[b] |
| RBC pHi | 7.431 ± 0.037 | 7.352 ± 0.051 | 7.428 ± 0.041 | 7.380 ± 0.026 |
| $\Delta H^+m$ (mequiv/L) | 3.90 ± 0.55[a] | 4.12 ± 0.80[a] | 2.15 ± 0.60[a] | 1.20 ± 0.35[a,b] |
| MCHC (g/mL) | 0.234 ± 0.017[a] | 0.221 ± 0.018[a] | 0.231 ± 0.007[a] | 0.271 ± 0.018[b] |
| Relative plasma volume (%) | 74.4 ± 2.2[a] | 70.3 ± 2.0[a] | 76.1 ± 1.9[a] | 92.2 ± 5.6[b] |
| Cortisol (ng/mL) | 322.0 ± 51.3[a] | 265.9 ± 47.8[a] | 292.2 ± 73.4 | 215.2 ± 39.1[a] |

[a]Significant change ($p < 0.05$) with respect to the control measurements taken from these same fish in the exposure water prior to challenge, as tabulated in Table 2. By definition, the control value for $\Delta H^+m$ is 0 and for the relative plasma value is 100%.

[b]Significantly different ($p < 0.05$) from comparable value of fish exposed to pH = 6.5, $Ca^{2+}$ = 25 μequiv/L, Al = 0 μg/L.

urements clearly indicated that this was the case (Fig. 2). Lactate did not change when naive fish were challenged with acid only (Fig. 2A), but increased in concert with falling $Pa_{O_2}$ during combined acid/Al challenge (Fig. 2B; cf. Fig. 1). This lactate mobilization was not appreciably altered by either higher water $Ca^{2+}$ levels (Fig. 2C) or prior exposure to low pH alone (Fig. 2D), but was prevented in the fish previously exposed to both stressors (Fig. 2E), confirming the protective action of this treatment.

Acid plus Al challenge induced a significant blood acidosis in fish naive to both stressors at $Ca^{2+}$ = 25 μequiv/L; pHa fell by ~0.25 unit (Table 3). In terms of standard acid–base terminology (Davenport 1974), this acidosis was of compound "respiratory" and "metabolic" origin, for $Pa_{CO_2}$ increased (respiratory component) and there was a significant accumulation of metabolic $H^+$ (i.e. positive $\Delta H^+m$). These two influences would be expected to exert opposing effects on plasma $HCO_3^-$, which did not change. Prior exposure and challenge at the higher $Ca^{2+}$ level slightly delayed but did not prevent these effects, although here the metabolic component was dominant, resulting in a significant fall in $HCO_3^-$ (Table 3). The final pHa in these fish was also substantially lower than in other groups (Table 3), but this reflected the fact that the starting pHa prior to challenge was also lower (Table 2); the actual fall in pHa was comparable. Prior exposure to acid alone did not ameliorate either the respiratory or metabolic components of acidosis during combined acid/Al challenge (Table 3). In contrast, the fall in pHa during challenge was entirely prevented by prior exposure to sublethal acid plus Al. In these fish, a significant respiratory alkalosis (fall in $Pa_{CO_2}$) counteracted a small metabolic acidosis ($\Delta H^+m$), resulting in unchanged pHa (Table 3). In general, measured increases in blood lactate (Fig. 2) were large enough to account for the metabolic component ($\Delta H^+m$) in all but the last group.

In contrast with extracellular pH (pHa), intracellular pH (pHi) in the RBC's was extremely well regulated during acid/Al challenge (Table 3). There were no significant variations at any time in RBC pHi, either within or between groups, despite substantial decreases in pHa and increases in $Pa_{CO_2}$ under some conditions.

In summary, these data provide physiological evidence that prior exposure to sublethal acid plus Al confers upon respiratory and acid–base functions almost complete acclimatory resistance to a more severe acid plus Al challenge for 72 h. The blockade of respiratory gas diffusion across the gills and accompanying compound acidosis seen in naive fish are clearly prevented. However, prior exposure to sublethal acid alone provides no such resistance. Higher water $Ca^{2+}$ levels slightly delay but do not prevent these deleterious respiratory and acid–base disturbances.

### Effects of Exposure Conditions on Other Physiological Responses to Challenge

In general, trends for other physiological parameters were in accord with those for respiratory and acid–base measurements. Thus, blood glucose, a sensitive stress indicator (Wedemeyer and McLeay 1981), did not change when naive fish were challenged with pH = 4.8 alone (Fig. 2A), but increased markedly in all three groups in which combined acid/Al challenge induced disturbances of gas exchange (Fig. 2B, 2C, 2D). Prior exposure to sublethal acid plus Al prevented the glucose response (Fig. 2E).

Plasma cortisol, another index of stress (Donaldson 1981), proved to be an even more sensitive indicator. Cortisol did not change over 66 h of exposure to pH = 4.8 alone (Fig. 3), but increased within 4 h of combined acid/Al challenge in naive fish at both low and high $Ca^{2+}$ levels. These increases were

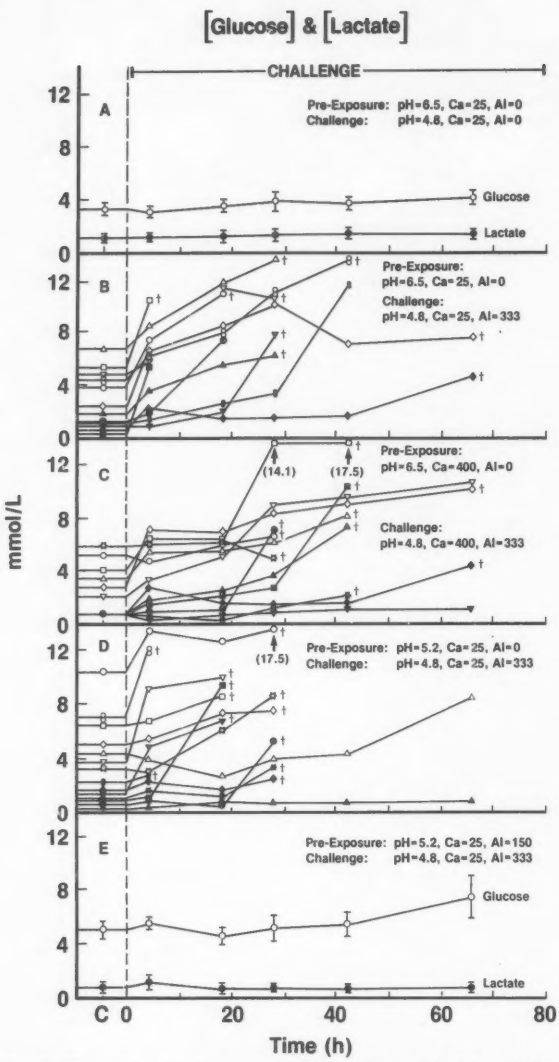


FIG. 2. Changes in glucose (open symbols) and lactate (solid symbols) concentrations of arterial blood in brook trout challenged for 3 d with acid alone (pH = 4.8) or acid plus Al (pH = 4.8, Al = 333 μg/L) in flowing artificial soft water. Other details as in legend to Fig. 1.

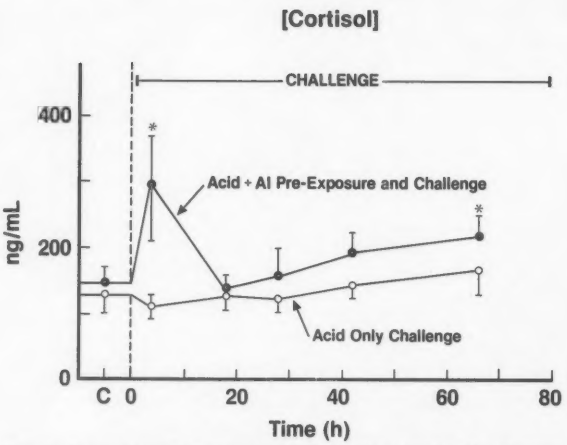


FIG. 3. Comparison of changes in plasma cortisol levels of brook trout under two different exposure/challenge treatments. Open symbols represent a group pre-exposed to pH = 6.5, $Ca^{2+}$ = 25 μequiv/L, Al = 0 μg/L for 10 wk and then challenged with pH = 4.8, $Ca^{2+}$ = 25 μequiv/L, Al = 0 μg/L. Solid symbols represent a group pre-exposed to pH = 5.2, $Ca^{2+}$ = 25 μequiv/L, Al = 150 μg/L for 10 wk and then challenged with pH = 4.8, $Ca^{2+}$ = 25 μequiv/L, Al = 333 μg/L. Means ± 1 SEM ($N$ = 6). Asterisks indicate means significantly different ($p < 0.05$) from the control mean taken from these same fish in their exposure waters prior to challenge.

maintained until the final samples (Table 3). Cortisol did not increase in the group previously exposed to sublethal low pH alone. However, this is not surprising inasmuch as cortisol levels were already elevated by a comparable amount in these fish as a result of the exposure itself, prior to challenge (Tables 2, 3). Interestingly, cortisol did increase initially during challenge in fish previously exposed to both stressors (Fig. 3). This rise was coincident with the transitory fall in $Pa_{O_2}$ at this time (4 h; Fig. 1E) and then disappeared as $Pa_{O_2}$ returned to control levels. However, by the final sample (66 h), cortisol was again elevated in this group (Fig. 3), suggesting that a second stress response was starting, perhaps triggered by ionoregulatory disturbance (see below).

Plasma $Na^+$ and $Cl^-$ levels fell slightly in naive fish challenged with pH = 4.8 alone, although the changes were significant only at the final sample (Fig. 4A). Challenge with acid plus Al provoked much more rapid decreases in both ions; $Cl^-$ fell to a greater extent than $Na^+$ (Fig. 4B), probably because of the accompanying rise in lactate (Fig. 2B). While fish at the higher water $Ca^{2+}$ level started with higher $Na^+$ and $Cl^-$ concentrations, this did not prevent comparable falls during acid/Al challenge (Fig. 4C). Prior exposure to sublethal acid alone was also ineffective in preventing this decline (Fig. 4D). Prior exposure to both acid and Al greatly attenuated the ionoregulatory disturbance, although plasma $Na^+$ and $Cl^-$ still decreased significantly at the final sample (Fig. 4E). Nevertheless, these changes were no more serious than those in naive fish exposed to acid alone (Fig. 4A).

There were no significant changes in plasma $Ca^{2+}$ as a result of challenge, but $K^+$ increased by ~50% in fish challenged with acid plus Al (data not shown). Again this response was not seen in the group previously exposed to both acid and Al.

Apparent shifts in fluid volume distribution also occurred during challenge. Plasma volume (as calculated from plasma protein changes; McDonald et al. 1980) did not change significantly in naive fish challenged with acid alone, but decreased by ~25% as a result of combined acid/Al challenge (Table 3). This contraction of plasma volume was not altered by either higher water $Ca^{2+}$ levels or prior exposure to pH = 5.2 alone, but was prevented by prior exposure to pH = 5.2, Al = 150 μg/L. MCHC, usually considered an index of RBC swelling (Milligan and Wood 1982), showed almost identical trends (Table 3). Thus there was an apparent fluid shift into the red cells in association with the decrease in plasma volume, an effect which was prevented by prior exposure to acid plus Al.

In summary, these data corroborate the conclusions based on respiratory and acid–base measurements and indicate that general stress responses, ionoregulatory problems, and fluid volume shifts occur in tandem with gas exchange and acid–base disturbances during acid/Al challenge. Therefore, acclimation

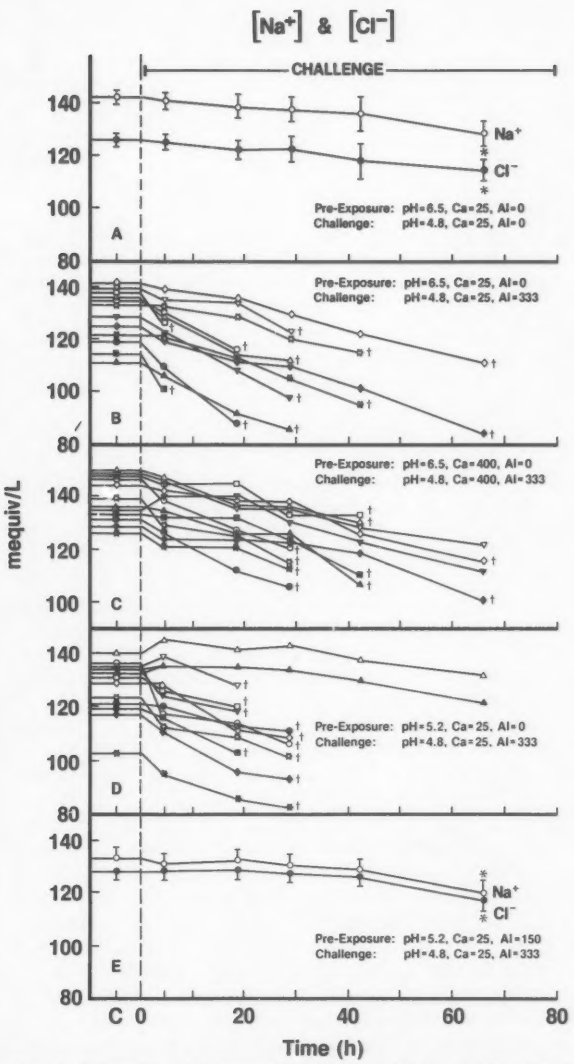


FIG. 4. Changes in the plasma $Na^+$ (open symbols) and $Cl^-$ (solid symbols) concentrations of arterial blood in brook trout challenged for 3 d with acid alone (pH = 4.8) or acid plus Al (pH = 4.8, Al = 333 μg/L) in flowing artificial soft water. Other details as in legend to Fig. 1.

to sublethal acid plus Al eliminates not just some, but virtually all short-term physiological responses to more severe combined challenge with the two stressors. Prior exposure to sublethal acid alone is ineffective, while higher $Ca^{2+}$ levels slightly delay but do not prevent the suite of toxic responses. Finally, the small disturbances in plasma $Na^+$, $Cl^-$ (Fig. 4E), and cortisol (Fig. 3) seen at 66 h in the acclimated fish suggest that acclimatory resistance may not last indefinitely.

## Discussion

### Effects of pH/$Ca^{2+}$/Al Exposures on Respiratory and Stress Indices

The blood gas, acid–base, and lactate measurements from cannulated fish (Table 2) demonstrated that 10 wk of sublethal acid or acid plus Al exposure had no chronic respiratory effect, at least in resting animals. Thus if there was a thickening of the lamellar epithelium which increased diffusive resistance of the gills (see the Introduction), it was compensated by other factors such as lamellar recruitment or ventilatory adjustments (cf. Wood and Perry 1985). However, it remains possible that the capacity for increasing $O_2$ and $CO_2$ exchange during exercise could have been reduced, for this was not tested. Acute ventilatory effects of acid and acid plus Al exposure have been documented (Hargis 1976; Neville 1979, 1985; Rosseland 1980; Walker et al. 1988), but there is little previous information on long-term responses. Giles et al. (1984) found a significantly elevated ventilation rate in rainbow trout (*Salmo gairdneri*) held for 22 d at pH = 4.9 or below, but interpretation was confounded by elevated $P_{CO_2}$ in their test waters (cf. Neville 1979). We are aware of no exercise studies on this topic.

The presence of elevated plasma cortisol and blood glucose after 10 wk of exposure to pH = 5.2, $Ca^{2+}$ = 25 μequiv/L, Al = 0 μg/L suggests that a chronic stress response occurred (Table 2). These findings are in general agreement with those of several studies on rainbow trout exposed to sublethal acidity (pH = 4.7 – 5.7) for 5 – 42 d (Brown et al. 1984; Barton et al. 1985; Brown et al. 1986a, 1986b) and lake whitefish (*Coregonus clupeaformis*) held at pH = 4.1 – 5.0 for 14 d (Scherer et al. 1986). In contrast, Lee et al. (1983) found no change in cortisol in rainbow trout maintained at pH's as low as 4.1 for 14–21 d, but they measured the hormone by a fluorometric technique, rather than by radioimmunoassay.

A unique finding of the present study is that the presence of Al = 150 μg/L at this same pH = 5.2, $Ca^{2+}$ = 25 μequiv/L prevented this stress response (Table 2). This further supports our contention, previously argued on the basis of plasma ion measurements only, that low levels of Al may actually be beneficial during chronic acid exposure (Wood et al. 1988a). Again, we speculate that this could result from either greater chloride cell proliferation (Tietge et al. 1988) leading to more effective recovery or a stabilizing action of $Al^{3+}$ on branchial permeability (Schofield and Trojnar 1980). However, it must also be noted that Al was not protective but rather detrimental to the growth of the present fish under acidic conditions (Mount et al. 1988).

A number of other parameters sampled by cannulation in the present study (plasma ions, protein, and hematology) duplicated those sampled by caudal puncture from a wider range of exposures in Wood et al. (1988a). In general, there was good agreement between the two studies, and the same explanations should apply. However, plasma $Ca^{2+}$ was substantially higher in the presence of higher water $Ca^{2+}$ in the present study, a difference which was not seen in the caudal puncture study. While this effect is not unexpected, it could have been exaggerated had there been a disproportionate number of females in the group, for vitellogenesis was just starting by the time of these experiments (D. R. Mount, unpubl. results). Unfortunately, the animals were not sexed in the present study.

### Responses to Challenge in Naive Fish

Qualitatively, the physiological responses of cannulated Wyoming brook trout to challenge with pH = 4.8 alone or pH = 4.8, Al = 333 μg/L resembled those of similarly exposed Ontario fish, and mortality rates were virtually identical (Wood et al. 1988b). This agrees with the results of ion flux studies on noncannulated fish which also showed comparable acid/Al

sensitivities in the two populations (Booth et al. 1988; Wood et al. 1988a). Thus in both groups, the effects of pH = 4.8 alone were negligible. In contrast, the effects of pH = 4.8 plus 333 μg/L Al were traumatic, involving ionic depletion, inhibition of respiratory gas exchange, hemoconcentration, acid–base dysfunction, and death. The causative mechanisms have been discussed in detail by Wood et al. (1988b); in brief, they all relate to the interaction of Al with the branchial epithelium.

Quantitatively, there were two notable differences in the responses of the Wyoming fish. Firstly, the extent of respiratory disturbance upon combined acid/Al challenge at $Ca^{2+}$ = 25 μequiv/L (Fig. 1, 2; Table 3) was much larger and comparable with that seen in Ontario fish at $Ca^{2+}$ = 400 μequiv/L. The most likely explanation is the difference in inspired $Po_2$ (~120 versus ~150 Torr) resulting from the difference in altitude (2200 versus 100 m), although differences in fish stocks or water quality cannot be excluded. The other discrepancy was in blood glucose, which increased greatly in the Wyoming fish prior to death (Fig. 2), but not in the Ontario fish. Glucose mobilization has not been assessed in previous studies on Al, but has been widely documented in fish acutely exposed to severe acid stress (e.g. McDonald 1983; Lee et al. 1983; Brown et al. 1984, 1986b; Barton et al. 1985; Scherer et al. 1986). The difference is probably due to the fact that the Ontario fish were starved for at least 7 d prior to test, while the Wyoming fish were fed up until the day of cannulation.

Two measurements unique to the present study were RBC pHi and plasma cortisol. RBC pHi was remarkably invariant in the presence of extracellular acidosis during acid plus Al challenge (Tables 2, 3). It is now clear that increased plasma catecholamines during acidotic stress in fish may play a critical role in stabilizing RBC pHi, thereby protecting the intracellular milieu of Hb for $O_2$ transport (Nikinmaa 1986). We therefore suggest that significant catecholamine mobilization occurred in the present fish. Circulating catecholamine levels have not yet been reported in any acid or acid/Al study on fish, although strong pharmacological evidence exists that they are elevated during severe acid challenge in rainbow trout (Milligan and Wood 1982).

Corticosteroids are the other group of hormones commonly mobilized during stress (Wedemeyer and McLeay 1981; Donaldson 1981). Rapid increases in plasma cortisol levels during acute, severe acid exposure (pH = 4.0 – 4.5) have been documented in brook trout (Mudge et al. 1977; Ashcom 1979) and rainbow trout (Adams et al. 1985), but at higher pH's (>4.7), the response took more than a week to develop in rainbow trout (Brown et al. 1984). This is in accord with the present results, where there was no increase in cortisol over 66 h of acute exposure to pH = 4.8 (Fig. 3) but a doubling after 10 wk of chronic exposure to pH = 5.2 (Table 1). Additionally, the present results show that the presence of Al (333 μg/L) at pH = 4.8 induces a rapid rise in plasma cortisol during acute exposure. We have seen a similar response in rainbow trout acutely exposed to Al = 112 μg/L at pH = 4.8 (Goss and Wood 1988). A possible benefit of cortisol mobilization in these circumstances may be its documented effect in stimulating chloride cell proliferation on the secondary lamellae (Doyle and Epstein 1972; Perry and Wood 1985).

### Effects of Exposure Conditions on Responses to Challenge

The present results show clearly that 10 wk of exposure to sublethal acid plus Al (pH = 5.2, $Ca^{2+}$ = 25 μequiv/L, Al = 150 μg/L) induces acclimatory resistance to a more severe acid plus Al challenge, while exposure to sublethal acid alone (pH = 5.2, $Ca^{2+}$ = 25 μequiv/L, Al = 0 μg/L) does not. This reinforces the conclusion with respect to Al acclimation of the accompanying investigation (Wood et al. 1988a), which was based only on $Na^+$ flux and plasma ion data. The present data additionally demonstrate protection against respiratory problems, acidosis, and hemoconcentration, as well as attenuation of the stress response signalled by glucose and cortisol elevation. The results also clarify the situation (i.e. lack of acclimation) with respect to the effect of prior exposure to acid alone, which had been equivocal previously, and show that higher water $Ca^{2+}$ levels slightly delay but do not prevent the toxic responses to acid plus Al challenge. Note that these experiments did not test whether prior exposure to acid alone induced greater resistance to challenge with acid alone, which is a separate issue.

These experiments provide physiological confirmation of the LC50 data of Orr et al. (1986) on rainbow trout who demonstrated that prior exposure to low levels of Al (87 and 154 μg/L at pH ≃ 5.2) provides increased resistance to higher levels (~1.8-fold increase in LC50 from ~175 to ~315 μg/L at pH ≃ 5.2 for both exposures). Our studies covered approximately the same range of concentrations, but with a more resistant species (Grande et al. 1978). Fish chronically exposed to low levels of Al in the wild will clearly possess greater resistance to short-term increases in Al associated with episodic events (snowmelt and rainstorm runoff). However, it is unclear whether this increased resistance represents true tolerance, i.e. whether it will allow the animals to survive indefinitely under the more severe condition. The results of Orr et al. (1986) suggest that it should, for they continued their tests for 6 d and were able to document increases in "incipient" LC50 levels, implying an enhancement of long-term survival. However, our Al-acclimated fish, while showing an absence or rapid correction of any initial disturbances during challenge, did exhibit the apparent start of a stress response at 66 h, manifested as decreased plasma $Na^+$ and $Cl^-$ (Fig. 4E) and elevated cortisol levels (Fig. 3). Much longer challenges will be required to test whether the increased resistance is of finite duration.

We have suggested that the protection against ion loss offered by Al acclimation could involve a greater uptake capacity of the proliferated chloride cells or a more effective reduction of passive permeability during acid/Al challenge (Wood et al. 1988a). It is difficult to see how such mechanisms could also prevent interference with $O_2$ and $CO_2$ diffusion across the gills. Perhaps the explanation is a more general one in that chronic sublethal exposure alters the nature of gill surface ligands for Al and/or the pH of the branchial microenvironment, so as to reduce Al accumulation during the challenge. This in turn might prevent mucification, inflammation, epithelial separation, and the associated increase in diffusion distance. We hypothesize that Al-acclimated trout accumulate less Al on the gills during a more severe challenge and that the morphological response of the branchial epithelium is attenuated. These topics are currently under investigation in our laboratories.

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# Sodium Transport in the Brook Trout, *Salvelinus fontinalis*: Effects of Prolonged Low pH Exposure in the Presence and Absence of Aluminum

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McDonald, D. G., and C. L. Milligan. 1988. Sodium transport in the brook trout, *Salvelinus fontinalis*: effects of prolonged low pH exposure in the presence and absence of aluminum. Can. J. Fish. Aquat. Sci. 45: 1606–1613.

This study examined the effects of prolonged exposure (≥10 wk) to low $Ca^{2+}$ (25 versus 400 μequiv/L), low pH (pH 5.2 versus 6.5) with and without Al (150 μg/L) on $Na^+$ uptake in adult brook trout (*Salvelinus fontinalis*). $Na^+$ uptake ($J_{in}^{Na+}$) was assessed by measuring radiotracer disappearance from the medium at ambient $Na^+$ (79 μequiv/L) and in response to acute increases in ambient $Na^+$. The relationship between $J_{in}^{Na+}$ and $[Na^+]$ was best described by a linear model with the slope of the line a measure of transport activity. Transport activity increased as a result of prolonged low $Ca^{2+}$ exposure at pH 6.5, but declined in fish maintained at pH 5.2. These fish showed no compensation in response to low pH; there was no recovery in transport activity when pH was acutely raised to 6.5, and lower pH's further inhibited uptake. In contrast, the $Na^+$ transport activity of fish maintained at pH 5.2 and 150 μg Al/L was significantly greater than that of fish acclimated to pH 5.2 alone and indeed greater than pH 6.5 controls. This indicates that Al exposure induced a compensatory mechanism in the gills that was not seen with low pH exposure alone.

On a examiné les effets, sur la captation du $Na^+$, d'une exposition prolongée (≥10 sem) d'ombles de fontaine (*Salvelinus fontinalis*) adultes à de faibles teneurs en $Ca^{+2}$ (25 versus 400 μequiv/L) et à un faible pH (pH 5,2 versus 6,5) en présence et en absence d'Al (150 μg/L). On a quantifié la captation du $Na^+$ ($J_{in}^{Na+}$) en mesurant le taux de disparition du milieu d'un indicateur radioactif à une teneur ambiante de $Na^+$ (79 μequiv/L) et après de rapides augmentations de la teneur ambiante en $Na^+$. Un modèle linéaire dont la pente de la courbe concordait à une quantification de l'activité de transport décrivait mieux la relation entre $J_{in}^{Na+}$ et $[Na^+]$. L'activité de transport a augmenté par suite d'une exposition prolongée à une faible teneur en $Ca^{+2}$ à un pH 6,5, mais a diminué chez les poissons gardés au pH 5,2. Ces derniers n'ont montré aucune réaction de compensation au faible pH; l'activité de transport ne s'est pas rétablie quand le pH a été rapidement porté à 6,5, tandis que de plus faibles pH ont entraîné une inhibition supplémentaire de la captation. Par contre, l'activité de transport du $Na^+$ chez les poissons gardés au pH 5,2 en présence de 150 μg/L d'Al était nettement plus élevée que chez les poissons acclimatés au pH 5,2 seulement et les poissons témoins gardés au pH 6,5. Ceci révèle que l'exposition à l'Al induit un mécanisme de compensation dans les ouïes qui n'est pas observé chez les individus exposés à un faible pH.

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It is well established that ion losses caused by disruption of branchial ionoregulation are central to the acid and acid/Al toxicity syndromes in fish (see reviews by Leivestad 1982; Wood and McDonald 1982; McDonald 1983; Wood 1988; McDonald et al. 1988). In a previous study on brook trout (*Salvelinus fontinalis*) (Booth et al. 1988), we confirmed earlier observations on salmonid fishes, showing that lower water pH's bring about net $Na^+$ losses across the gills because uptake is inhibited and efflux stimulated (Packer and Dunson 1970; McWilliams and Potts 1978; McDonald et al. 1983). We also demonstrated that Al, at least acutely, is additive to the effects of $H^+$, further inhibiting influx and further stimulating efflux.

Although the effects of low pH on gill function, particularly in ion regulation, have received considerable attention in the last 10 yr, there are still some important questions left unresolved: What are the effects on the gills of long-term sublethal exposure to low pH? Do the gills accumulate damage with continued exposure, rendering the fish more susceptible to lethal exposures (i.e. sensitization), or can the fish restore normal gill ionoregulatory function (transport and ion permeability) in the presence of the stressor (i.e. recovery)? Does, in fact, the recovery process convey increased tolerance of more severe acid/Al exposures (i.e. acclimation)? Further, while our initial studies (Booth et al. 1988) showed that Al and $H^+$ are additive to one another, are there, in fact, qualitatively different responses to these two stressors? Our other studies in this series (Wood et al. 1988a, 1988b) suggest that brook trout are able to adapt to sublethal acid/Al stress, but their responses to acid/Al exposure are qualitatively different from those to low pH alone. The source of these adaptive responses may well be at the gills, given the primacy of this organ as the target of Al/$H^+$ toxicity.

Thus the purpose of this study was to examine the adaptive responses of the $Na^+$ transport mechanism in the brook trout

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under continuous, long-term exposures to constant pH, $Ca^{2+}$, and Al conditions. Specific objectives were, firstly, to characterize the $Na^+$ transport mechanism in brook trout at circumneutral pH (pH 6.5) in terms of kinetics and sensitivity to changes in water $Ca^{2+}$, secondly, to evaluate fully the acute responses of the transport mechanism to pH reduction and to $Al/H^+$ exposure and thirdly, to establish whether prolonged exposure to either acid alone or in combination with Al has any effect on $Na^+$ transport activity beyond that which appears as an acute effect, i.e. whether any sensitization, recovery, or acclimation has occurred.

## Materials and Methods

Experiments were conducted at the Fish Physiology and Toxicology Laboratory, University of Wyoming. Adult brook trout were obtained from Cline's Trout Farm (Boulder, Colorado) and were maintained prior to experimentation for at least 10 wk in flowing water with 1 of 12 possible combinations of pH (6.5 or 5.2), $Ca^{2+}$ (25 or 400 μequiv/L), and Al (0, 75, or 150 μg/L), as described in detail in Wood et al. (1988a). A subset of these animals, 80 in total averaging 263 ± 6 g each, were used for the experiments of this study. During the month it took to perform these experiments the average $[Na^+]$ in the water was 79 ± 5 μequiv/L.

### Experimental Protocol

In this study we have employed a new "divided-box" technique in measuring branchial ion fluxes. This technique is new in the sense that it does not require anaesthetization or surgical immobilization to restrain the fish and yet is able to reduce the volume of water bathing the gills by as much as possible without causing undue stress. The advantage gained is the ability to make accurate measurements of fluxes over very short time periods without contributions from other parts of the body including the kidneys.

Fish were transferred from their tanks of origin to a rectangular opaque Plexiglas chamber (65 cm wide × 39 cm long × 6 cm deep), which consisted of a "head" and "tail" section divided by an inflatable rubber collar. Inflation of the collar gently restrained the fish caudal to the pectoral fins, thereby sealing its head in a chamber of 480-mL nominal volume (actual volume was lower because of displacement by the fish). The fish was further gently restrained by means of longitudinal plates placed on either side of the body in the tail section. The head chamber volume was recirculated by means of a Cole–Parmer centrifugal pump at 500 mL/min past an aerator and a pH electrode. Water pH was maintained within 0.01 pH unit by means of a pH stat system, consisting of a Radiometer PHM 84 pH meter, Cole–Parmer combination pH electrode, and a Radiometer TTT80 titration controller controlling a motor-driven 2.5-mL Gilmont micrometer burette filled with 0.1 N HCl. The partial pressure of oxygen in water ($Po_2$) was periodically monitored and the aerator adjusted to ensure that $Po_2$ remained within 90% of air saturation. Water temperature was maintained at 10 ± 0.5°C by immersing the flux chamber in a water bath. A 10- to 15-min adjustment period was allowed to elapse prior to the start of an experiment during which time the head and tail chambers were flushed with water of the same composition as the animal's tank of origin. In preliminary studies using fish cannulated in the caudal artery (Wood et al. 1988b), we confirmed that blood $Po_2$ and acid–base status remained at normal levels while the fish was restrained in the divided box. From this we conclude that the confinement stress was minor. During the experiment, water samples, typically of 5 mL, were periodically taken from the head chamber and an equal volume of water from the animal's tank of origin was replaced at that time. At the termination of the experiment, the volume of the head chamber was determined by dye dilution. This also served to check for any leaks of the rubber collar.

### Experiments

*1. $Na^+$ uptake kinetics*

$Na^+$ transport activity at constant pH was assessed by examining the relationship between $Na^+$ uptake ($J_{in}^{Na^+}$) and $[Na^+]$. These experiments were 72 min in duration following the initial 10- to 15-min adjustment period. $[Na^+]$ was increased from ambient (50–100 μequiv/L) to 1800 μequiv/L in three successive steps. Each step, including the initial step at ambient $Na^+$, was of 16-min duration, of which the first 4 min was used as a mixing period. In the first step, only radiotracer ($^{22}Na^+$, 0.2 μCi (7.4 kBq)) was added to the water; subsequently, $Na^+$ (as NaCl) was added along with radiotracer, 0.2 μCi $^{22}Na^+$/100 μequiv $Na^+$. Water samples (5 mL) were taken at 4 and 16 min of each step and subsequently analyzed for $[Na^+]$, $^{22}Na^+$ activity, and ammonia concentration. The volume of acid added by the micrometer burette in maintaining constant pH was periodically recorded and used in the calculation of the net flux of acidic equivalents (see below).

*2. Acute effect of pH reduction*

These experiments were either 104 or 82 min in duration depending on the starting pH (i.e. either pH 6.5 or 5.2). The initial measurements were always at the pH of the animal's tank of origin in water of similar ionic composition except that [NaCl] was raised from acclimation levels (70 μmol/L) to either 150 or 650 μmol/L. The former was used to increase the accuracy of $Na^+$ influx measurements at low pH, and the latter was used to test whether elevated $[Na^+]$ would alter the response to low pH. In each experiment, water pH was reduced as many as four steps until pH 4.4 was reached. In the first step, at the acclimation pH, radiotracer (0.5 μCi of $^{22}Na^+$) was added to the water and allowed to mix for 4 min and a water sample was collected immediately and again 12 min later. The titrator was then adjusted to the next lower pH and further radiotracer added (0.5 μCi). The titrator achieved the new pH within 2 min, but 10 min was allowed to elapse before the next water sample was collected to ensure complete titration of the chamber contents and thorough mixing of the radiotracer. At each successive pH the mixing period was 10 min and the flux measurement period was 12 min.

*3. Acute effect of Al exposure*

These experiments were 106 min in duration and consisted of a 28-min control period (4-min radiotracer mixing period and two 12-min flux measurement periods in water from the animal's tank of origin), a 42-min Al exposure period (6-min titration/mixing period and three 12-min flux periods at pH 4.8 and 333 μg Al/L), and a 36-min recovery period (12-min flush with acclimation water and two 12-min flux measurement periods). Al/low pH exposure was achieved by resetting the titrator to pH 4.8 and by addition of Al stock (60 mg Al/L as $AlCl_3$) to yield an [Al] of 333 μg/L. At the end of Al exposure and prior to the recovery measurements the head chamber was flushed for 12 min with water from the animal's tank of origin and the titrator reset to the pH of this water.

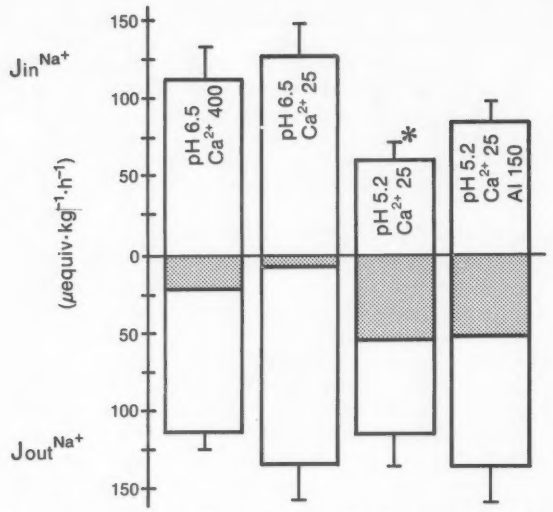


FIG. 1. $Na^+$ uptake and efflux ($J_{in}^{Na^+}$ and $J_{out}^{Na^+}$) in brook trout at 10°C measured under various holding conditions (pH, $[Ca^{2+}]$, and [Al] as noted) and at ambient $[Na^+]$. Data are means ± 1 SEM, $N = 6$. $[Na^+]$ was 88 (4.6), 70 (7.3), 73 (5.2), and 74 (9.3) μequiv/L (mean, SEM in parentheses), respectively, for the four holding conditions. Shaded areas indicate net flux ($J_{net}^{Na^+}$). Asterisk indicates that $J_{in}^{Na^+}$ at pH 5.2, zero [Al] was significantly less than at pH 6.5 by unpaired *t*-test ($p < 0.05$).

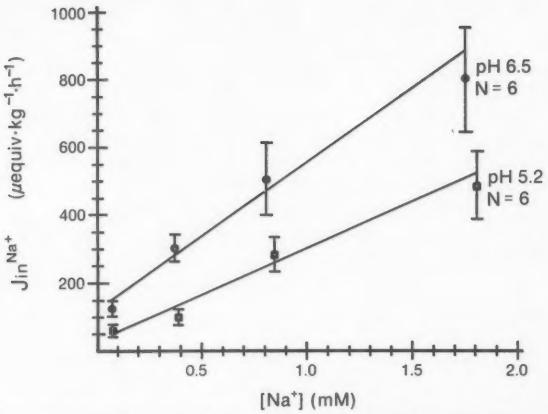


FIG. 2. Interrelationship between $Na^+$ uptake ($J_{in}^{Na^+}$) and external $[Na^+]$ for two holding conditions: pH 6.5 and pH 5.2 in low-$Ca^{2+}$ water (25 μequiv/L). Lines fitted by least squares regression: pH 6.5, $Y = 0.384\,X + 141.2$ ($r = 0.990$); pH 5.2, $Y = 0.252\,X + 34.8$ ($r = 0.988$).

### Analytical Procedures

$Na^+$ influx ($J_{in}^{Na^+}$ in microequivalents per kilogram per hour) was determined according to the following formula:

$$(1)\quad J_{in}^{Na^+} = \frac{cpm_i - cpm_f \cdot vol\ (mL)}{time\ (h) \cdot wt\ (kg) \cdot SA}$$

where cpm is the concentration of radiotracer ($Na^{22}$) in the medium (counts per minute per millilitre) at the beginning (i) and end (f) of the measurement period, vol is the volume of the headchamber (millilitres) determined by dye dilution, SA is the radiospecific activity of the external medium (counts per minute per microequivalent), and wt is the animal's body weight (kilograms).

$Na^+$ net flux ($J_{net}^{Na^+}$ in microequivalents per kilogram per hour) was determined from changes in $Na^+$ concentration in the water, and $Na^+$ efflux ($J_{out}^{Na^+}$) was determined by difference:

$$(2)\quad J_{out} = J_{in} - J_{net}.$$

The net flux of acidic equivalents ($J_{net}^{H^+}$ in microequivalents per kilogram per hour) was determined (cf. McDonald and Wood 1981) as the difference between the net flux of ammonia ($J_{net}^{AM}$) determined from changes in water ammonia concentration and the net flux of titratable alkalinity ($J_{net}^{TA}$). The latter was determined from the volume of 0.1 N HCl added to the flux chamber to maintain a constant pH according to the following formula:

$$(3)\quad J_{net}^{TA} = \frac{Vol\ of\ titrant\ (\mu L) \cdot N\ (\mu equiv/\mu L)}{time\ (h) \cdot wt\ (kg)}.$$

$[Na^+]$ in the water was determined on a Perkin–Elmer atomic absorption spectrophotometer (model 2380). Ammonia concentration was determined by a micromodification of the salicylate–hypochlorite assay of Verdouw et al. (1978).

### Statistical Analysis

Means ± 1 standard error of the mean (SEM) are reported throughout. Student's two-tailed *t*-test (unpaired design) was used to assess significant differences ($p \leq 0.05$) between groups. Least squares regression analysis was used to evaluate the relation between $[Na^+]$ in the water and $Na^+$ uptake. Significant differences between slopes were determined by *t*-test.

## Results

### Characteristics of $Na^+$ Transport in Brook Trout at pH 6.5

Fish maintained at pH 6.5 and examined at the $[Na^+]$ of their tanks of origin (79 ± 5 μequiv/L) were essentially in balance, i.e. the rate of $Na^+$ uptake across their gills ($J_{in}^{Na^+}$) was virtually the same as the rate of $Na^+$ loss ($J_{out}^{Na^+}$) (Fig. 1). Net fluxes of $Na^+$ ($J_{net}^{Na^+}$, shaded areas in Fig. 1) were not significantly different from zero in either those animals maintained in low $Ca^{2+}$ or those maintained in high $Ca^{2+}$ (25 or 400 μequiv/L) at circumneutral pH (Fig. 1). There were no significant differences between the two groups, although influx and efflux values were slightly higher in the low-$Ca^{2+}$ group.

Increasing the water $[Na^+]$ from ambient levels to nearly 2000 μequiv/L caused an increase in $J_{in}^{Na^+}$ in all animals, as would be expected, but over the range tested there was no evidence of saturation of the $Na^+$ transport system. Indeed, the relationship between uptake and external concentration was either linear or only slightly curvilinear (Fig. 2). Consequently, the data do not allow the more typical analysis of $Na^+$ uptake, i.e. transport kinetics expressed in terms of maximum uptake rate ($V_{max}$) and carrier affinity ($k_m$) (cf. Maetz 1974). Instead, transport activity was evaluated in terms of the slope of the linear regression equation relating uptake to concentration (i.e. $J_{in}^{Na^+} = \text{slope} \cdot [Na^+] + \text{intercept}$). Here, the units of transport activity are microequivalents per kilogram per hour per microequivalent, i.e. $Na^+$ uptake per increment in external

TABLE 1. Summary of $Na^+$ kinetic measurements on brook trout at 10°C. Data are means ± 1 SEM. Units for [$Ca^{2+}$] and [Al] are μequiv/L and μg/L, respectively. Data for plasma $Na^+$ are from Wood et al. (1988a, $N = 6$) from the same groups of fish used in the present study. $Na^+$ transport activity is the slope of the regression equation: $J_{in}^{Na+} = a \cdot [Na^+] + b$. Net $Na^+$ gain is the sum of $J_{net}^{Na+}$ values from each ambient $Na^+$ (i.e. from 0.08 to 1.8 mmol/L). Asterisks indicate means significantly different from mean at pH 6.5 and 25 μequiv $Ca^{2+}$/L ($p < 0.05$, unpaired $t$-test).

| Holding conditions | | | Measurement conditions | $N$ | $Na^+$ transport activity (μequiv·kg$^{-1}$·h$^{-1}$·μequiv$^{-1}$) | Avg. $Na^+$ efflux (μequiv·kg$^{-1}$·h$^{-1}$) | Net $Na^+$ gain (μequiv/kg) | Plasma [$Na^+$] (mequiv/L) |
|---|---|---|---|---|---|---|---|---|
| pH | [$Ca^{2+}$] | [Al] | | | | | | |
| 6.5 | 400 | 0 | Same | 6 | 212 ± 35* | 141 ± 42 | 115 ± 28* | 148 ± 2* |
| 6.5 | 25 | 0 | Same | 6 | 384 ± 39 | 158 ± 47 | 291 ± 79 | 138 ± 1 |
| 5.2 | 25 | 0 | Same | 6 | 252 ± 28* | 142 ± 58 | 92 ± 49* | 141 ± 2 |
| 5.2 | 25 | 0 | pH 6.5 | 6 | 140 ± 26* | 161 ± 50 | 67 ± 36* | 141 ± 2 |
| 5.2 | 25 | 150 | Same | 5 | 464 ± 31 | 170 ± 82 | 189 ± 43 | 141 ± 1 |

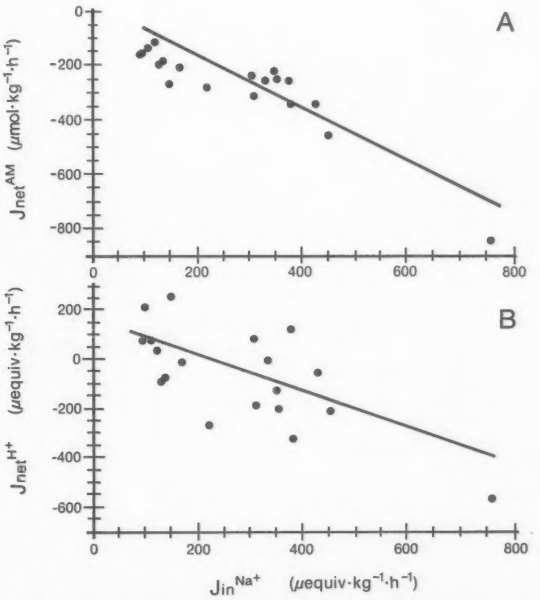


FIG. 3. Correlations between (A) $Na^+$ uptake ($J_{in}^{Na+}$) and net ammonia excretion ($J_{net}^{AM}$) and (B) $J_{in}^{Na+}$ and net flux of acidic equivalents ($J_{net}^{H+}$) at pH 6.5. Regression lines based on data from $N = 6$ fish (4 from 400 μequiv $Ca^{2+}$/L and 2 from 25 μequiv $Ca^{2+}$/L). Lines fitted by least squares regression: $J_{net}^{AM} = -0.871\,(J_{in}^{Na+}) - 29.2$ ($r = 0.900$); $J_{net}^{H+} = -0.841\,(J_{in}^{Na+}) + 165.8$ ($r = 0.708$).

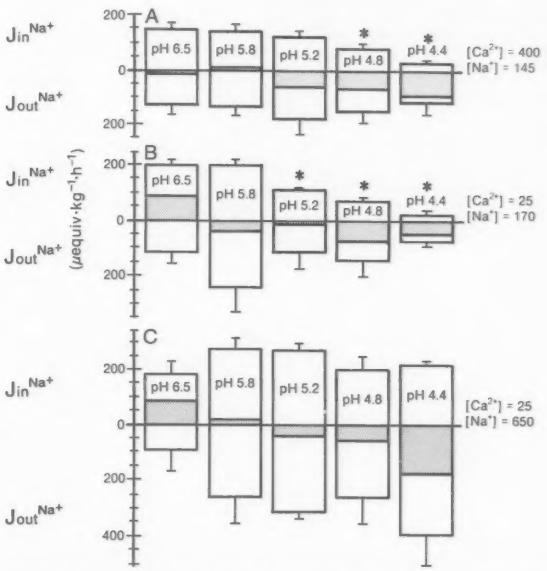


FIG. 4. Acute effect of pH on $Na^+$ fluxes in brook trout maintained at pH 6.5. Data are means ± 1 SEM; open bars indicate $J_{in}$ and $J_{out}$ and shaded bars indicate $J_{net}$. Fish were held at each pH for 22 min, 10 min for pH equilibration and 12 min for measurement. (A) $N = 7$, fish maintained at 400 μequiv $Ca^{2+}$/L, [$Na^+$] set to 145 μequiv/L; (B) $N = 5$, fish maintained at 25 μequiv $Ca^{2+}$/L, [$Na^+$] set to 170 μequiv/L; (C) $N = 4$, fish maintained at 25 μequiv $Ca^{2+}$/L, [$Na^+$] set to 650 μequiv/L. Asterisks indicate $J_{in}$ significantly different from $J_{in}$ at pH 6.5 (by unpaired $t$-test, $p < 0.05$).

[$Na^+$]. The transport activities calculated in this manner are listed in Table 1.

Despite the apparent lack of effect of $Ca^{2+}$ on $J_{in}^{Na+}$ at ambient $Na^+$ levels (Fig. 1), animals maintained at reduced $Ca^{2+}$ (i.e. 25 versus 400 μequiv/L) at circumneutral pH (pH 6.5) showed a significant increase in $Na^+$ transport activity. In these animals, transport activity was nearly double that of animals maintained in high $Ca^{2+}$ (384 ± 39 versus 212 ± 35 μequiv·kg$^{-1}$·h$^{-1}$·μequiv$^{-1}$) (Table 1).

Although increasing [$Na^+$] caused increased $J_{in}^{Na+}$, $J_{out}^{Na+}$ remained virtually constant in each of the test groups (Table 1). Furthermore, the average $J_{out}^{Na+}$ was relatively constant amongst the test groups, varying between 140 and 170 μequiv·kg$^{-1}$·h$^{-1}$. Since $J_{out}^{Na+}$ was constant, the increase in $J_{in}^{Na+}$ with increasing external [$Na^+$] resulted in a significant net $Na^+$ gain by all animals. In high-$Ca^{2+}$ animals, this led to a gain of $Na^+$ of 115 ± 28 μequiv/kg (Table 1). In low-$Ca^{2+}$ animals, this gain was significantly higher (291 ± 79 μequiv/kg) because of their higher transport activity.

Concomitant measurements of ammonia excretion ($J_{net}^{AM}$) and the net flux of acidic equivalents ($J_{net}^{H+}$) reveal that $J_{in}^{Na+}$ was fairly closely correlated, at pH 6.5, with $J_{net}^{AM}$ ($r = 0.900$, Fig. 3A) and with $J_{net}^{H+}$ ($r = 0.708$, Fig. 3B). The former relationship has a slope of near unity, suggesting a nearly 1:1 exchange between $Na^+$ and $NH_4^+$. However, the correlation between $J_{in}^{Na+}$ and $J_{net}^{H+}$ suggests that $Na^+/H^+$ exchange could also be occurring. In any case, the measurement of $J_{net}^{H+}$ does not distinguish between the fluxes of $H^+$ and $NH_4^+$, and both are equivalent in terms of the acid–base status of the animal.

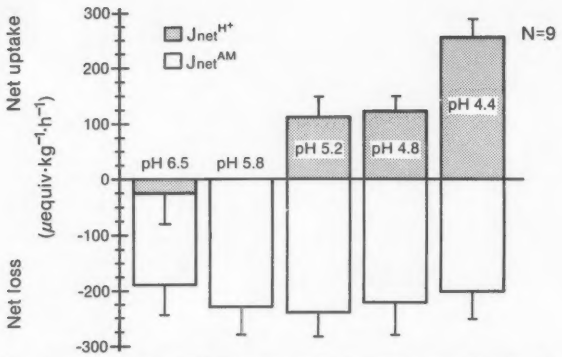


FIG. 5. Acute effect of pH reduction on net ammonia excretion ($J_{net}^{AM}$, open bars) and net flux of acidic equivalents ($J_{net}^{H+}$, shaded bars) in brook trout acclimated to pH 6.5. Data are means ± 1 SEM ($N$ = 9, 5 fish maintained at 400 μequiv $Ca^{2+}$/L and 4 maintained at 25 μequiv $Ca^{2+}$/L). Fish were held at each pH for 22 min, 10 min for pH equilibration and 12 min for measurement. A large net uptake of acidic equivalents was recorded at pH 5.8, but this was not plotted, as it likely was an artifact of the titration of residual $HCO_3^-$ in the water.

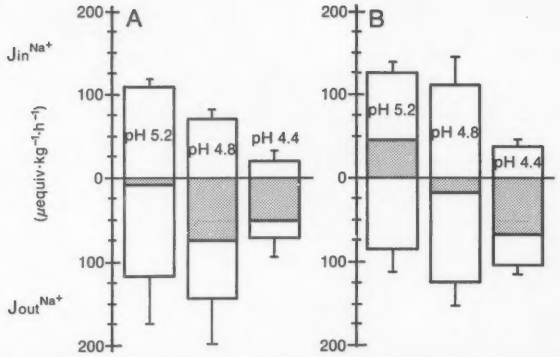


FIG. 6. Acute effects of pH reduction on $Na^+$ fluxes in brook trout maintained in low-$Ca^{2+}$ water (25 μequiv/L) at (A) pH 6.5 and (B) pH 5.2, $N$ = 5 and 6, respectively. Data are means ± 1 SEM; open bars indicate $J_{in}$ and $J_{out}$, and shaded bars indicate $J_{net}$. Fish were held at each pH for 22 minutes, 10 min for pH equilibration and 12 min for measurement. Water [$Na^+$] was 190 ± 40 μequiv/L.

## Effects of Low pH on $Na^+$ Transport

In these experiments, fish were held 16 min at each pH level. The main effect of acute pH reduction was inhibition of $Na^+$ uptake (Fig. 4). The inhibition was significant at pH ≤ 4.8 in high-$Ca^{2+}$ animals (Fig. 4A) and at pH ≤5.2 in low-$Ca^{2+}$ animals (Fig. 4B). In both groups, $J_{out}^{Na+}$ was not significantly stimulated by pH reduction and indeed was significantly reduced in the low-$Ca^{2+}$ group at pH 4.4 relative to pH 6.5. The overall result in both groups was a progressive increase in net $Na^+$ loss with decreasing pH. There were no significant differences between the groups.

When this experiment was repeated at a substantially higher [$Na^+$] (650 versus 150 μequiv/L, [$Ca^{2+}$] = 25 μequiv/L), the effects of water pH were markedly altered (Fig. 4C). Here, there was no inhibition of $J_{in}^{Na+}$ even at pH 4.4. Nonetheless, there were similar or greater net $Na^+$ losses relative to the fish at lower ambient [$Na^+$] (Fig. 4A, 4B) because $J_{out}^{Na+}$ increased progressively with pH reduction, in marked contrast with fish at the lower ambient $Na^+$.

Ammonia excretion averaged about 200 μmol·$kg^{-1}$·$h^{-1}$ and was unaffected by reduction in water pH (Fig. 5). In contrast, $J_{net}^{H+}$ was markedly affected. At pH 6.5, there was a slight net loss of acidic equivalents which was not significantly different from zero. Reduction of pH led to a net uptake of acidic equivalents which increased progressively with decreasing pH.

In fish maintained at pH 5.2 for about 10 wk and examined at the [$Na^+$] of their tanks of origin (73 ± 5 μequiv/L) (Fig. 1), $J_{in}^{Na+}$ averaged 60 ± 11 ($N$ = 7). This is significantly lower than $J_{in}^{Na+}$ at pH 6.5 ($t$-test, $p < 0.05$) and is also lower than $J_{in}^{Na+}$ of fish acutely exposed to pH 5.2 (109 ± 9 μequiv·$kg^{-1}$·$h^{-1}$, Fig. 4B). In contrast, $J_{out}^{Na+}$ of fish maintained at pH 5.2 was nearly identical to that of fish at pH 6.5 (Fig. 1).

As a result of their lower than normal $J_{in}^{Na+}$, fish maintained at pH 5.2 experienced net $Na^+$ loss, at least during the measurement period (FIg. 1). However, these losses are unlikely to have remained at this level in their tank of origin, since the fish would ultimately lose a fatal amount of $Na^+$. Furthermore, the plasma [$Na^+$] in these fish was not significantly different from that of fish maintained at pH 6.5, low $Ca^{2+}$ (Table 1). Rather, these losses suggest that fish maintained at pH 5.2 were more sensitive to the handling and confinement stress attendant to the flux measurements than were fish maintained at pH 6.5

Measurements of $Na^+$ transport in relation to ambient [$Na^+$] further indicated the persistent inhibitory effect of maintaining fish at pH 5.2 (Table 1). The activity of $Na^+$ transporter in these fish (i.e. the slope of $J_{in}$ versus [$Na^+$] plot, Fig. 2) was about 65% of that of pH 6.5, low-$Ca^{2+}$ fish. Although pH 5.2 fish went from a net loss of $Na^+$ (Fig. 1) to net uptake when ambient [$Na^+$] was elevated, with their lower transport activity, their net $Na^+$ gain was less than half compared with the pH 6.5 group (Table 1).

To test whether the reduction in transport activity in pH 5.2 fish was simply due to the lower water pH, transport activity was examined in this group upon acute exposure to pH 6.5. Here, the transport activity was not enhanced by the return of pH to circumneutral levels. Indeed, both the activity and the net gain of $Na^+$ of this group were substantially less at pH 6.5 than at pH 5.2 (Table 1). To test the nature of the effect, pH 5.2 fish were acutely exposed to lower pH's (Fig. 6). Lower pH exposure further inhibited $Na^+$ uptake (Fig. 6B) while having little affect on $J_{out}^{Na+}$. Compared with fish maintained at pH 6.5 (Fig. 6A), $J_{in}^{Na+}$ was less affected at pH 4.8 and net losses much smaller in pH 5.2 fish. However, at pH 4.4 the pH 5.2 and pH 6.5 groups showed virtually identical degrees of inhibition of $J_{in}^{Na+}$.

## Effect of Al on $Na^+$ Transport

Three groups of fish were acutely exposed to an elevated Al concentration, 333 μg/L at pH 4.8. The three groups were fish that had been maintained in 400 μequiv $Ca^{2+}$/L at pH 6.5 (Fig. 7A), pH 5.2 (Fig. 7B), and pH 5.2 and 150 μg Al/L (Fig. 7C). Although exposure to 333 μg Al/L induced net $Na^+$ losses in all groups, Al had little effect beyond that produced by pH 4.8 alone. Fish from pH 6.5 showed a greater degree of inhibition of $J_{in}^{Na+}$ at 4.8/333 compared with 4.8 alone (broken lines, Fig. 7A) but less stimulation of $J_{out}^{Na+}$. Consequently, net losses of $Na^+$ were similar for the two treatments. A similar phenomenon was observed in pH 5.2 fish (broken lines, Fig. 7B). For fish maintained at pH 5.2/150 μg Al/L the acute

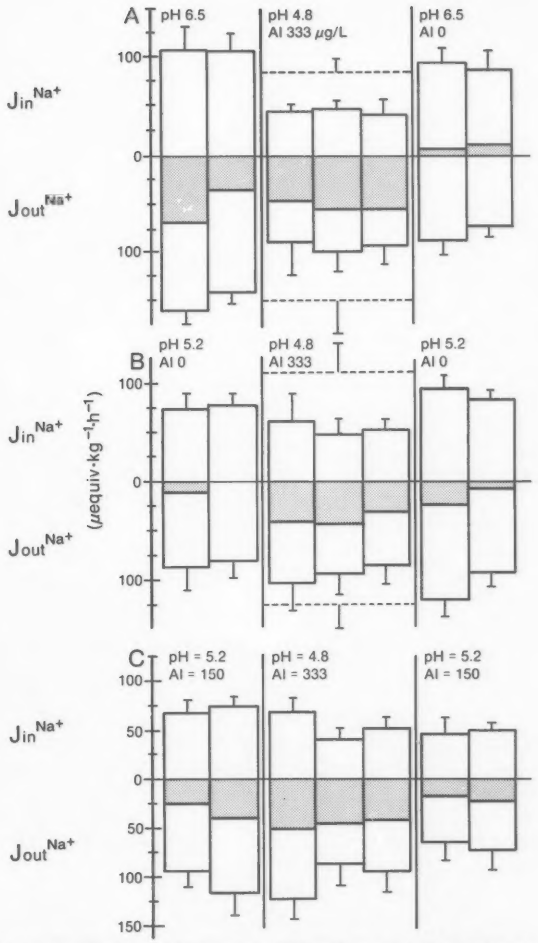


FIG. 7. Acute effect of Al exposure (333 μg/L at pH 4.8) on fish maintained at (A) pH 6.5 ($N$ = 5), (B) pH 5.2, zero Al ($N$ = 5), and (C) pH 5.2, 150 μg Al/L ($N$ = 6). Data are means ± 1 SEM; open bars indicate $J_{in}$ and $J_{out}$ and shaded bars indicate $J_{net}$. Each bar represents a 12-min measurement period. The broken lines in the middle panel indicate the effect on $J_{in}$ and $J_{out}$ of exposure to pH 4.8 alone (data are means ± 1 SEM taken from Fig. 4 and 6).

exposure to 4.8/333 produced no significant change in either $J_{in}$ or $J_{out}$ (the effect of pH 5.2 alone was not tested on these fish). Furthermore, the groups showed virtually identical fluxes during the 48-min Al exposure period, and each returned immediately to control flux rates upon restoration of their respective control conditions.

## Discussion

### Characteristics of $Na^+$ Transport in Brook Trout

The kinetics of $Na^+$ transport have been examined in a number of studies on freshwater fish (Table 2). Without exception, these studies have demonstrated that $Na^+$ transport shows saturation or Michaelis–Menten type kinetics within the $[Na^+]$ range 0–3 mmol/L. These findings have been interpreted as indicating that $Na^+$ transport is mediated by a carrier having a limited number of transport sites and therefore describable in terms of a maximum transport rate ($V_{max}$) and an apparent affinity constant ($k_m$), the $[Na^+]_{ext}$ at which the flux is half-maximal (Maetz 1974).

In contrast, our results show an apparent absence of saturation kinetics at least over the range of 0 – 2 mmol NaCl/L. One possible interpretation is that $Na^+$ transport is not carrier mediated in brook trout. However, a simpler and more likely explanation is that the carrier has such a low affinity that saturation kinetics would not be apparent over the range of external $Na^+$ tested. The absence of saturation kinetics makes quantitative comparisons between the brook trout and other species difficult. To enable a comparison, Table 2 lists $J_{in}^{Na+}$ values for each study calculated or measured at 1.8 mmol/L (the highest $[Na^+]$ employed in the present study). This analysis suggests that the $Na^+$ transport capacity of the brook trout is not unusual relative to other fish examined.

When an aquatic animal is faced with an ion-poor environment, there are potentially three strategies the animal might use to preserve salt balance (cf. Kirschner 1980): increase carrier affinity (i.e. lower $k_m$), increase transport capacity (increase in number of transport sites, $V_{max}$), and/or decrease ion permeability. In rainbow trout (*Salmo gairdneri*), the data in Table 2 suggest that their principal strategy is to increase carrier affinity (i.e. reduce $k_m$). Indeed, the $k_m$ value reported by Wood and Randall (1973) for rainbow trout adapted to soft Vancouver tap water ($[Na^+]$ = 40, $[Ca^{2+}]$ = 240 μequiv/L) is the lowest value recorded for any freshwater vertebrate. In contrast, the strategy apparently adopted by the brook trout is one of reduction of ion permeability, since apparently the affinity is low and the transport activity is not unusually high relative to the other species. Nonetheless, transport activity increased substantially in brook trout with reduction in $Ca^{2+}$ even though $[Na^+]$ was held constant. The likeliest interpretation of this finding is that ion leakiness of the gills increased with reduction in $Ca^{2+}$ from 400 to 25 μequiv/L, necessitating an increase in transport rate. Note, as well, that plasma $[Na^+]$ levels were depressed in low-$Ca^{2+}$ animals relative to high-$Ca^{2+}$ animals (Table 1). This further supports the view that ion permeability had increased. These observations underline the critical role played by external $Ca^{2+}$ in controlling the stability and integrity of gill epithelia (McDonald and Rogano 1986).

In addition to fundamentally different strategies for adapting to low-ion media, another apparent difference between the brook trout and the rainbow trout is the occurrence in the latter of an increase in $J_{out}^{Na+}$ with increasing $J_{in}^{Na+}$ (Kerstetter et al 1970; Wood and Randall 1973). This $Na^+/Na^+$ exchange, which plays apparently no useful role in ion balance and is measurable only with radiotracer, has been interpreted either as a property of the carrier, i.e. "a leaky pump" (a pump mechanism requiring a counter-ion to operate and where internal $Na^+$ can replace $H^+$ as the counter-ion (after Maetz 1974)), or a property of a leaky epithelium; at low external $[Na^+]$, much of the outward leak of ions by simple diffusion would be "back-transported" by an otherwise unoccupied carrier (Kirschner 1955). Therefore, at higher ambient $[Na^+]$, $J_{out}$ would increase as more external $Na^+$ occupied the carrier. Thus the absence of $Na^+/Na^+$ exchange in the brook trout (Table 1) could either be interpreted as a fundamentally different transport mechanism or could simply be a reflection of a generally lower ionic permeability in the brook trout relative to the rainbow trout. Certainly, a difference in the intrinsic

TABLE 2. Comparison of $Na^+$ transport in freshwater fish. Units for $k_m$ and $V_{max}$ are μequiv/L and μequiv·kg$^{-1}$·h$^{-1}$, respectively. Ambient [$Na^+$] is the environment that the animals were maintained in prior to measurement.

| Species | Ambient [$Na^+$] (μequiv/L) | $k_m$ | $V_{max}$ | $J_{in}$[a] | Reference |
|---|---|---|---|---|---|
| *Platichthys flesus* | 200 | 800 | 350 | 244 | Maetz 1971 |
| *Carassius auratus* | 100 | 300 | 650 | 557 | Maetz 1972 |
| *Salmo gairdneri* | 900 | 450 | 333 | 266 | Kerstetter et al. 1970 |
| *Salmo gairdneri* | 595 | 57 | 540 | 523 | Lauren and McDonald 1987a |
| *Salmo gairdneri* | 70 | 20 | 617 | 610 | Wood and Randall 1973 |
| *Salvelinus fontinalis* | 79 | — | — | 794 | $Ca^{2+}$ = 25, this study |
| *Salvelinus fontinalis* | 79 | — | — | 440 | $Ca^{2+}$ = 400, this study |

[a]$J_{in}$ values at 1800 μequiv/L were calculated from the Michaelis–Menten equation (cf. Maetz 1974): $J_{in} = (J_{max}\cdot[Na^+]_{ext})/(k_m + [Na^+]_{ext})$.

branchial ion permeability would explain the well-known differences in acid sensitivity between these two species (Grande et al. 1978).

Despite these species differences, we can confirm that in brook trout, as previously demonstrated in rainbow trout (Kerstetter et al 1970) and goldfish (*Carassius auratus*) (Maetz 1972), $Na^+$ uptake occurs in exchange for an endogenous cation, either $NH_4^+$ or $H^+$, since we show a clear correlation between their respective fluxes (Fig. 3). The observation of the link between $J_{in}^{Na^+}$ and the net flux of acidic equivalents is interesting, since it can only occur if the $Cl^-$ exchange mechanism ($Cl^-$ uptake in exchange for excretion of basic equivalents, $OH^-$ or $HCO_3^-$; cf. Maetz et al. 1976) is operating at a lower intensity (if both exchangers were operating at a similar intensity, $J_{net}^{H^+}$, the sum of the unidirectional fluxes of $H^+$, $NH_4^+$, $OH^-$, and $HCO_3^-$, would necessarily have to be near zero). Other studies support the view that $Cl^-$ transport is of generally lower intensity than $Na^+$ transport in freshwater fish (cf. McDonald and Rogano 1986). In any case, these observations suggest that the $Cl^-$ transport mechanism in brook trout would be worthy of detailed investigation.

## Effects of Prolonged Exposure to Low pH and Al

In the present study, the various alterations in $Na^+$ transport activity occurring in response to low pH or Al exposure suggest that brook trout acclimated to sublethal Al (at low pH) but not to low pH alone. Animals maintained at 150 μg Al/L at pH 5.2 had $Na^+$ transport activities comparable with those of animals maintained at pH 6.5 whereas animals at pH 5.2 alone showed significantly less activity. Indeed, the latter exhibited an even greater degree of inhibition of uptake after 10 wk at pH 5.2 than animals acutely exposed to pH 5.2 (Fig. 4). This confirms and extends our previous observation on brook trout (Booth et al. 1988) of lack of recovery of $Na^+$ uptake with 10 d of acid exposure. Furthermore, this inhibition is not simply a continued competitive interaction by $H^+$ for the $Na^+$ transport mechanism, for when the pH 5.2 exposed animals were measured at pH 6.5, there was no improvement in their transport activity (Table 1). The implication is that the transport mechanism was damaged in some way by continued acid exposure. This conclusion parallels those of the toxicity studies which have shown little if any increased resistance of fish to lethal pH exposure following prolonged sublethal pH exposure (see McDonald 1983 for review) whereas a recent study by Orr et al. (1986) showed clear evidence of acclimation to Al. Indeed, acclimation to other metals has also been reported (Zn, Cu, Cd, Hg) (Chapman 1978; Dixon and Sprague 1981; Pascoe and Beattie 1979; Duncan and Klaverkamp 1983). Recent work in our laboratory with Cu (Lauren and McDonald 1987b) has shown that the phenomenon is centred upon synthesis of the transport protein $Na^+$-$K^+$-ATPase in the gills. The mechanism of Al acclimation may be similar, since the principal acute effects of Cu are similar to Al in causing severe disturbance to branchial ionoregulation (Lauren and McDonald 1986). Therefore, it is quite possible that a synthesis of transport proteins is responsible for the increased $Na^+$ transport seen in Al-exposed fish (Table 1). The finding of acclimation to metals but not to $H^+$ suggests that the chemical interactions of $H^+$ with the gill epithelium must be fundamentally different from those of trace metals. The nature of these interactions has recently been examined in some detail (McDonald et al. 1988), but no definitive statements can yet be made. Much is still to be learned of the microenvironment of the gill surface and subsurface and of its biochemical responses to environmental stressors.

The response of low pH exposed fish to elevated external $Na^+$ further reveals something of the nature of the effects of low pH (Fig. 4C). Although the high $Na^+$ effectively prevented the inhibition of $J_{in}^{Na^+}$, no significant protection resulted because $J_{out}^{Na^+}$ also increased. The simplest interpretation for this finding is that at acid pH's $J_{out}$ and $J_{in}$ are linked, i.e. $Na^+$/$Na^+$ exchange. Although we present evidence that $Na^+$/$Na^+$ exchange does not take place at circumneutral pH in brook trout, it may be that it does appear in the epithelium when the pH is reduced. If this is the case, it adds further support to the notion that $Na^+$/$Na^+$ exchange is a function of the permeability of the epithelium (see above), since an increase in permeability is one of the principal initial effects of low pH exposure (McDonald et al. 1983; McDonald 1983).

## Acute Effect of Al Exposure

In the present study there was very little acute effect of 333 μg Al/L at pH 4.8 (Fig. 7). Based on our previous studies, we expected a greater response, since the exposure is moderately severe, causing significantly greater ion losses than pH 4.8 alone, an eightfold higher $J_{out}^{Na^+}$, and also some mortalities (Booth et al. 1988, fig. 4; Wood et al. 1988a, fig. 3 and 4). Part of the explanation for the difference is the higher $Ca^{2+}$ level employed in the present study (400 versus 25 μequiv/L), but the main reason is likely one of time. In the present study, Al exposure lasted only 48 min, while in our previous studies

we typically allowed 2–8 h to elapse before measuring fluxes. Furthermore, our Wyoming studies indicate that ion losses continue to increase for up to 24 h after the start of Al exposure (Wood et al. 1988a). This temporal delay in the effect of Al is not a property of low pH exposure. As near as we can tell, the effects of low pH are immediate and are certainly within minutes; the inhibition of $J_{in}$ seen after less than 30 min of low pH exposure (Fig. 4, 6) was virtually the same as that seen after 8 h (Booth et al. 1988, fig. 8). The contrast between Al and $H^+$ once again emphasizes that while they have qualitatively similar effects on branchial ion regulation, they quite clearly operate by fundamentally different mechanisms, permitting temporal delay and ability to acclimate with one (Al) but causing immediate effects and no recovery with the other ($H^+$).

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# Effects of Low pH and Aluminum on Ventilation in the Brook Trout (*Salvelinus fontinalis*)

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Walker, R. L., C. M. Wood, and H. L. Bergman. 1988. Effects of low pH and aluminum on ventilation in the brook trout (*Salvelinus fontinalis*). Can. J. Fish. Aquat. Sci. 45: 1614–1622.

Brook trout (*Salvelinus fontinalis*) (acclimated to pH = 6.5, $Ca^{2+}$ = 400 μequiv·$L^{-1}$), when exposed to acid (pH = 4.8, $Ca^{2+}$ = 400 μequiv·$L^{-1}$) and Al (333 μg·$L^{-1}$), responded with a twofold increase in ventilation volume within the first 4 h of the challenge period (100 h). Increased ventilation stroke volume accounted for most of the change in ventilatory response; rate increased slightly. Although ventilation volume returned to prechallenge values by 6 h, coughing (flow reversal) and increased mucus production at the gills were notable throughout the challenge period. There were no significant changes in oxygen consumption or $Pa_{O_2}$, but hemoglobin oxygen content (micromoles per gram of hemoglobin) decreased by 20%. Arterial pH decreased as a result of both respiratory and metabolic disturbances. Exposure to acid (pH = 4.8, $Ca^{2+}$ = 400 μequiv·$L^{-1}$) in the absence of Al resulted in similar initial changes in ventilation and blood acid–base status; however, ventilation remained elevated above the prechallenge values throughout the experiment (24 h). The transient increase and subsequent return of ventilation to prechallenge levels in the acid/Al-exposed fish suggests that Al interfered with the mechanism controlling the ventilatory response.

Des ombles de fontaine (*Salvelinus fontinalis*) acclimatés (pH = 6,5; $Ca^{+2}$ = 400 μequiv/L) et exposés à un milieu acide (pH = 4,8; $Ca^{+2}$ = 400 μequiv/L) et à de l'Al (333 μg/L) ont réagi par une augmentation double du volume de ventilation au cours des quatre premières heures de la période de provocation (100 h). Une augmentation du volume sistolique a expliqué la plus grande partie de la variation de la réaction ventilatoire, soit une légère augmentation du taux. Quoique le volume de ventilation soit revenu à sa valeur pré-provocation après 6 h, le renversement du débit et l'augmentation de la production de mucus au niveau des ouïes étaient évidents pendant toute la période de provocation. Aucune variation significative de la consommation d'oxygène ou du $Pa_{O_2}$ n'a été observée, mais la teneur en oxygène de l'hémoglobine (micromoles par gramme d'hémoglobine) a diminué de 20 %. Des perturbations respiratoires et métaboliques ont entraîné une baisse du pH artériel. L'exposition à un milieu acide (pH = 4,8, $Ca^{+2}$ = 400 μequiv/L) en absence d'Al a entraîné des variations initiales semblables dans le cas de la ventilation et de l'équilibre acidobasique du sang; toutefois, le taux de ventilation est demeuré élevé et supérieur aux valeurs pré-provocation pendant toute la durée de l'expérience (24 h). L'augmentation momentanée et le retour ultérieur du taux de ventilation au niveau pré-provocation chez les poissons exposés à un milieu acide et à l'Al portent à croire que l'Al perturbe le mécanisme qui contrôle la réaction ventilatoire.

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Trout hyperventilate in response to the combined effects of acid and Al (Muniz and Leivestad 1980; Rosseland 1980; Neville 1985). This observation was made by measuring opercular rate in unrestrained brown trout (*Salmo trutta*) and brook trout (*Salvelinus fontinalis*) (Muniz and Leivestad 1980; Rosseland 1980) or cough rate and mouth gape in juvenile rainbow trout (*Salmo gairdneri*) (Neville 1985). Ventilation volume was not measured in any of these studies. The response depends on the pH and $Ca^{2+}$ concentration of the water. Below pH 4.5 and at $Ca^{2+}$ concentrations less than 250 μequiv·$L^{-1}$, the combined effect of acid and Al was most severe (Rosseland 1980; Neville 1985). Mucus clogging of the gills and reduction of oxygen extraction from the water were also reported.

Blood data in accord with these findings have been reported by Wood et al. (1988b, 1988c) for brook trout exposed to a variety of acid, $Ca^{2+}$, and Al conditions. Severe respiratory and ionoregulatory disturbances were attributed to interaction of Al with the branchial epithelium. Gas exchange appeared to be greatly reduced, resulting in hypoxemia and hypercapnia in some treatment groups.

The effects on ventilation of acidification of water in the absence of Al are less clear. Dively et al. (1977) reported increases in mouth gaping and opercular expansion within 15–30 min and increased ventilation rate for the next 2–3 d of acid exposure. Neville (1985) reported similar ventilatory effects within the first hour of exposure of juvenile rainbow trout to

soft water at pH 4.5. However, Neville (1979) suggested that the initial increase in ventilation seen with acidification of water can be attributed to hypercapnia rather than an increase in plasma proton concentration. Janssen and Randall (1975) reported that a reduction in water pH to 5.0 caused a slow gradual increase in ventilation in rainbow trout, probably related to a slowly developing hypoxemia due to gill mucification. Wood et al. (1988b, 1988c), on the other hand, reported no significant disturbances in blood respiratory, acid–base, or ionoregulatory parameters in brook trout exposed to acid (pH 4.8) in the absence of Al.

The purpose of this study was to investigate the effects of acid versus acid plus Al on ventilation, oxygen uptake, blood respiratory gases, and acid–base status of brook trout acclimated to soft water. The effects of Al in the absence of acid could not be tested because Al is virtually insoluble in water at neutral pH. A $Ca^{2+}$ level (400 $\mu$equiv·$L^{-1}$) towards the upper end of the softwater range was chosen because Wood et al. (1988b) found that higher $Ca^{2+}$ exacerbated problems in respiratory blood gas regulation in Ontario brook trout under combined acid plus Al exposure. In light of the observations of Wood et al. (1988b, 1988c), it was hypothesized that the combined exposure to acid plus Al would greatly increase ventilation, both ventilation rate and stroke volume, and reduce the oxygen content of the blood, ultimately resulting in respiratory failure. The purpose of the acid-alone group was to clarify whether the ventilatory effects were attributable to the acid or the Al component of the challenge. Based on the results of Janssen and Randall (1975) and Wood et al. (1988b, 1988c), it was expected that there would be very little effect of acid on ventilation in the absence of Al. There have been no previous direct measurements of ventilation volume in fish subjected to acid/Al challenge.

## Materials and Methods

### Animal and Experimental Conditions

Experiments were conducted at the Fish Physiology and Toxicology Laboratory, Laramie, Wyoming (altitude = 2200 m). Brook trout weighing 150–350 g were acclimated for 10 wk to artificial soft water (pH = 6.5, $Ca^{2+}$ = 400 $\mu$equiv·$L^{-1}$) at 11 ± 1°C. Details regarding the generation of the artificial soft water are given in Wood et al. (1988a). In brief, the water was prepared from hard well water by sediment filtration, NaCl softening, reverse osmosis, and separate bed deionization (Continental Water Systems, Denver, Colorado). Following adjustment to pH 6.5 with KOH and the addition of $Na^+$ (40 $\mu$equiv·$L^{-1}$) and $Ca^{2+}$ (25 $\mu$equiv·$L^{-1}$) as chlorides, the water was thoroughly mixed and delivered to head tanks. From there it was distributed to 340-L fiberglass acclimation tanks at a rate of 1.9 L·$min^{-1}$. The $Ca^{2+}$ concentration was adjusted upward to 400 $\mu$equiv·$L^{-1}$ by addition of $CaCl_2$ via a marriott bottle to the acclimation tanks. Mixing was provided by aeration. Water pH was measured daily and other parameters ($Ca^{2+}$, $Na^+$, and $Cl^-$) weekly.

Fish were fed 1% body weight floating trout chow per day (Purina No. 5106) and solid wastes were removed by siphon daily. Photoperiod was adjusted biweekly to follow the natural cycle for Laramie, Wyoming.

Two groups of test animals were used: those challenged with acid and Al and those with acid in the absence of Al. A paired design was used with the prechallenge values for each fish acting as its own control.

The challenge consisted of either pH = 4.8, Al = 333 $\mu$g·$L^{-1}$ for up to 100 h (series I) or pH = 4.8, Al = 0 $\mu$g·$L^{-1}$ for 24 h (series II). Series II was conducted following completion of series I and was designed to test whether the initial increases in ventilation seen in series I were due to Al or simply the result of acidification of the water. Al (as $AlCl_3$) was added via marriott bottles to continuous-flow serial diluters and then to individual Plexiglas ventilation chambers (see below). The water pH was adjusted to 4.8 at the head tank by addition of $H_2SO_4$ using a Leeds and Northrup pH controller (model 7083).

Following the method outlined in Wood et al. (1988b), fish were anesthetized with MS-222 (ethyl *m*-aminobenzoate, 50 mg·$L^{-1}$; Sigma Chemical Co., St. Louis, Missouri). The water containing the anesthetic was adjusted to pH = 6.5 using 1 N NaOH and maintained at 11 ± 1°C using a glass cooling coil. Under anesthesia, fish were equipped with either caudal artery or vein catheters. Differentiation of successful arterial as opposed to venous catheterization was based upon observations of the blood pressure in the catheter following recovery from surgery. In addition, an oral membrane (to allow separation of inspired and expired water) was sutured around the mouth, as described by Davis and Cameron (1970). Each fish was placed in its own ventilation box which was supplied with acclimation water (pH = 6.5, $Ca^{2+}$ = 400 $\mu$equiv·$L^{-1}$) at a flow rate of 0.5 L·$min^{-1}$. Except for a hole in the top of the box, strategically placed to allow observation of opercular movements, the boxes were covered in black plastic to reduce disturbance.

The box was identical to that illustrated by Davis and Cameron 1970). In brief, the principle of the measurement is that the oral membrane separates the inspired from the expired water, the hydrostatic levels of which are maintained at identical values by constant level overflows. The inspired chamber is served with an excess water inflow. Timed collection of water passing out the overflow of the expired chamber represents direct measurement of the fish's ventilation volume. A Plexiglas tube placed around the fish body provides light restraint and serves to thoroughly mix the expired water. A length of P.E. 160 was carefully positioned inside the restraining tube posterior to the opercula to sample the expired water for $P_{O_2}$ determination. The water sample was obtained by siphoning (~0.5 mL·$min^{-1}$) into a 1-mL syringe with the plunger removed. The water was allowed to overflow the syringe by two to three volumes before the plunger was inserted and the syringe removed.

Following a 48-h recovery period, prechallenge ventilation rate ($f_R$, number per minute) was determined by observation of opercular movements, and ventilation volume ($\dot{V}_w$, millilitres per kilogram per minute) was measured by timed collection of water draining from the expired chamber of the ventilation box. Stroke volume ($V_s$, millilitres per kilogram per stroke) was calculated from ventilation volume and ventilation rate. Oxygen consumption (micromoles per kilogram per minute) was determined by the Fick method, using the difference in $P_{O_2}$ between the water of the inspired chamber and mixed expired water from just behind the operculae, the ventilation volume, and an assumed oxygen solubility of 2.23 $\mu$mol·$L^{-1}$·$Torr^{-1}$ at 10°C (Dejours 1975).

After measuring the ventilatory parameters while in the acclimation water, an initial blood sample was drawn from the catheter using an heparinized, ice-cold, gas-tight Hamilton syringe. The blood sample (400 $\mu$L) was replaced by reinfusing the blood recovered from the $P_{O_2}$ electrode (~100 $\mu$L) and

nonheparinized Cortland's saline (Wolf 1963). The blood was analyzed for arterial or venous $O_2$ tension ($Pa_{O_2}$ or $Pv_{O_2}$), pH (pHa or pHv), total $O_2$ in whole blood ($Ca_{O_2}$ or $Cv_{O_2}$), total $CO_2$ in true plasma ($C_{CO_2}$), hematocrit (Hct), and hemoglobin concentration ([Hb]).

A Radiometer G297/G2 electrode was used for the determination of pHa or pHv, and oxygen tension was measured with a Radiometer E5046 $P_{O_2}$ electrode. Both electrodes were maintained at 11°C. $C_{CO_2}$ was determined with a Corning 960 $CO_2$ analyzer. The true plasma was obtained from sealed, heparinized capillary tubes following centrifugation of whole blood at 5000 × *g* for 5 min. After Hct was read, the tube was broken at the plasma red cell interface and the plasma withdrawn with a Hamilton syringe for transfer to the Corning $CO_2$ analyzer.

Arterial $CO_2$ tension ($Pa_{CO_2}$) was calculated from the Henderson–Hasselbalch equation, using the $\alpha \cdot CO_2$ and pK values for trout at 11°C (Boutilier et al. 1984). True plasma bicarbonate concentration ($[HCO_3^-]$) was calculated using the formula

$$[HCO_3^-] = C_{CO_2} - (\alpha \cdot CO_2 \times Pa_{CO_2})$$

where $C_{CO_2}$ is the total $CO_2$ content and ($\alpha \cdot CO_2 \times Pa_{CO_2}$) represents the dissolved fraction. In the instances where $P_{CO_2}$ was measured, the method outlined by Boutilier et al. (1978) was employed using a Radiometer E5036 $P_{CO_2}$ electrode.

[Hb] was determined colorimetrically using the cyanmethemoglobin method (Sigma kit No. 525). Mean cell Hb concentration (MCHC) was calculated as the [Hb]/Hct ratio (i.e. grams Hb per millilitre of red blood cells).

The oxygen content of whole blood was determined using a Lex-$O_2$-Con oxygen analyzer (Lexington Instruments Corp.). After conversion of the oxygen content from volume percent to millimoles per litre, the blood oxygen content was calculated by subtracting away the dissolved component, using the solubility coefficient of oxygen in human plasma (Boutilier et al. 1984). To express the content in terms of [Hb] (micromoles oxygen per gram of Hb), the oxygen content (micromoles per litre) was divided by [Hb] (grams per litre).

Following the prechallenge sampling period, in the series I experiments, acid/Al water was piped into the anterior portion of the ventilation chamber. Ventilation was measured 30 min into the challenge and then again immediately prior to blood sampling at 1, 2, 4, 8 (or 9), 24, 48, 72, and 96 h of exposure, if death did not occur earlier. In series II, the fish were challenged with acid alone and sampled in a similar manner; however, the challenge was terminated at the end of 24 h of exposure and the fish were returned to the acclimation conditions.

### Treatment of Data

As discussed by Wood et al. (1988b, 1988c), it is difficult to accurately express group means when fish die at different times during the challenge. Therefore, in the case of the acid/Al group, the responses only during the first 20 h of the challenge have been portrayed as means ± 1 SEM. For those parameters where there were significant effects, the individual data were plotted. However, for all parameters, the mean ± 1 SEM of the prechallenge and final values are summarized, where the final values represent either the end of the challenge period or the last measurement prior to death. Since there were no mortalities in the acid-alone group, the mean values throughout the 24-h challenge period are plotted. In both groups a paired design was used; therefore, a Student's two-tailed *t*-test was employed for comparison between the prechallenge and intervening or final values. In all cases, statistical significance implied $p \leq 0.05$.

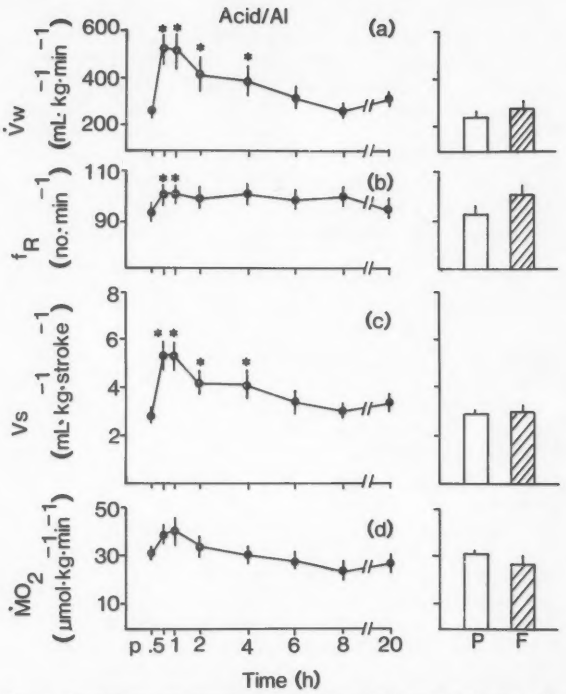


FIG. 1. Respiratory responses in brook trout challenged with acid (pH 4.8) and Al (333 μg·L$^{-1}$) in flowing water at 11°C following acclimation to pH 6.5 ($Ca^{2+}$ = 400 μequiv·L$^{-1}$ in both acclimation and challenge water). (a) Ventilation volume, $\dot{V}_w$; (b) ventilation rate, $f_R$; (c) ventilation stroke volume, $V_s$; (d) oxygen consumption, $\dot{M}_{O_2}$. The prechallenge values are indicated at "P" and by open bars. The final values (F, cross-hatched bars) were taken at 100 h or just prior to death. All points are means ± 1 SEM ($n$ = 11). Statistical significance ($p \leq 0.05$) between the prechallenge and challenge values is indicated by an asterisk.

## Results

Of 11 fish used in series I (acid/Al challenge), seven survived 20 h of acid/Al challenge and five were alive at 50 h. Only two survived past 60 h; of these, one died at 74 h whereas the other remained alive at the termination of the experiment at 100 h. This pattern of death and survival time was similar to that reported by Wood et al. (1987c) for identically challenged Wyoming brook trout which had been similarly cannulated but not fitted with oral membranes.

There was a significant increase in the ventilation volume ($\dot{V}_w$) within the first 30 min of exposure to pH 4.8 and 333 μg Al·L$^{-1}$ (from 258 to 516 mL·kg$^{-1}$·min$^{-1}$, Fig. 1). $\dot{V}_w$ remained above the prechallenge value for 4 h following the introduction of the acid/Al water. From 6 h to the end of the experiment, there was no significant difference in the mean $\dot{V}_w$ compared with the prechallenge period. Despite an increase in coughing frequency and mucus production in all fish, only three individ-

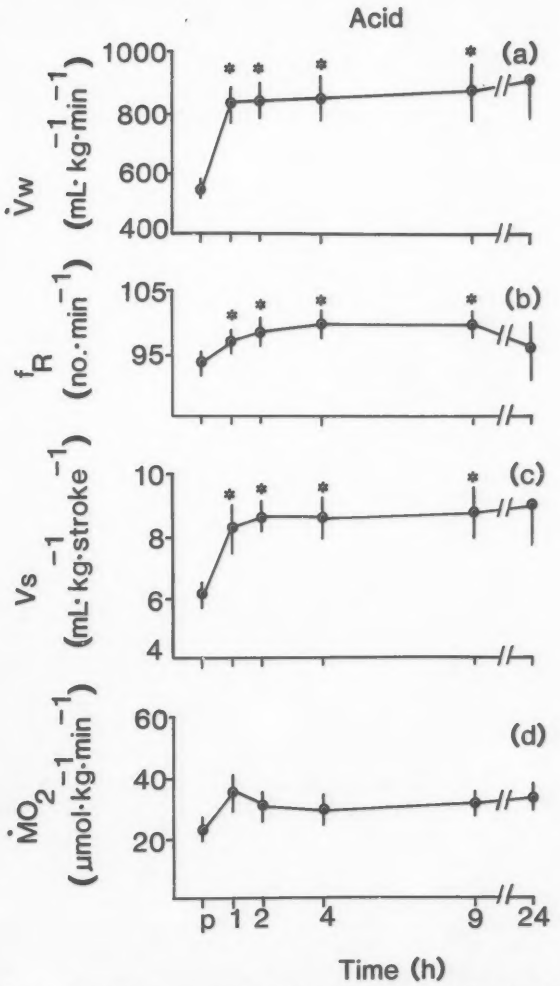


FIG. 2. Respiratory responses in brook trout challenged with acid (pH 4.8) in flowing water at 11°C after acclimation to pH 6.5 ($Ca^{2+}$ = 400 μequiv·$L^{-1}$ in both acclimation and challenge water), (a) Ventilation volume, $\dot{V}_w$; (b) ventilation rate, $f_R$; (c) ventilation stroke volume, $V_s$; (d) oxygen consumption, $\dot{M}_{O_2}$. The prechallenge values are indicated at "P." All points are means ±1 SEM ($n$ = 12). Statistical significance ($p \leq 0.05$) between the prechallenge and challenge values is indicated by an asterisk.

uals showed an increase in $\dot{V}_w$ prior to death, and as indicated in Fig. 1a, there was no significant difference between the average prechallenge and final $\dot{V}_w$.

The increase in $\dot{V}_w$ seen within the first 4 h can be mainly attributed to increased ventilation stroke volume ($V_s$, Fig. 1c). There was a rapid increase in $V_s$ within the first 30 min of exposure to the acid/Al mixture (2.87–5.37 mL·$kg^{-1}$·$stroke^{-1}$). $V_s$ remained elevated for 4 h and then declined to prechallenge levels. Opercular rate ($f_R$) was significantly elevated during the same time period (Fig. 1b); however, the relative increase (about 8% above preexposure values) was far less than that for $V_s$ (about 85% increase). There was no significant difference between the prechallenge and the final $f_R$ or $V_s$ (Fig. 1b and 1c).

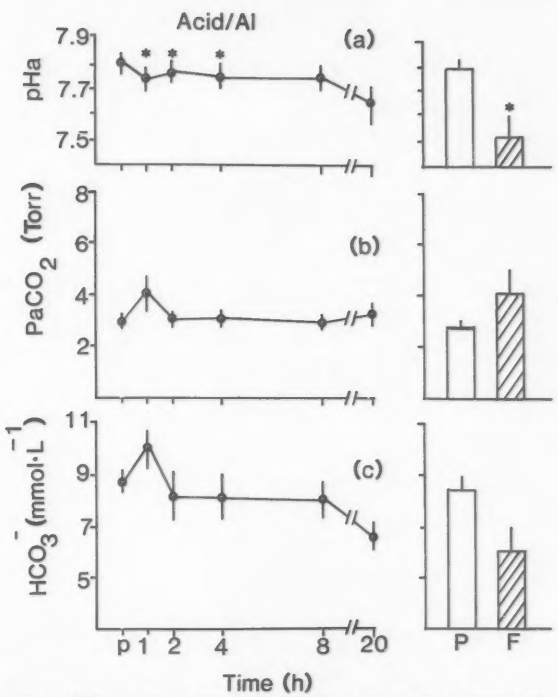


FIG. 3. Changes in the blood acid–base status of brook trout challenged with acid (pH 4.8) and Al (333 μg·$L^{-1}$) in flowing water at 11°C after acclimation to pH 6.5 ($Ca^{2+}$ = 400 μequiv·$L^{-1}$ in both acclimation and challenge water). (a) Arterial pH, pHa; (b) arterial $CO_2$ tension, $Pa_{CO_2}$ (calculated); (c) plasma bicarbonate concentration, $HCO_3^-$. All points are means ±1 SEM ($n$ = 7). Other details as in legend to Fig. 1.

Similar trends in ventilation were seen in the series II (acid-exposed) fish (Fig. 2). $V_s$ and $\dot{V}_w$ increased approximately 1.5-fold within the first hour, while there was little change in $f_R$. Unlike the acid/Al group, $V_s$ and $\dot{V}_w$ did not return to prechallenge values for the remainder of the measurement period (24 h). Also, $f_R$ continued to increase to 100·$min^{-1}$ by 4 h, which was significantly higher than the prechallenge mean. The fact that the prechallenge values for $\dot{V}_w$ and $V_s$ were significantly greater in the acid group than in the acid/Al group may reflect the difference in the body size of the two groups. The average weight of the acid/Al fish was 292 ± 17 g (SEM) and that of the acid-alone fish was 168 ± 7 g (SEM). The size difference was due to lack of availability of larger fish when the acid-alone group was tested.

Oxygen consumption ($\dot{M}_{O_2}$) tended to increase upon initial exposure to both acid and acid/Al (Fig. 1d and 2d), but the means were not significantly different from their respective prechallenge values. There was no significant difference between the average initial and final $\dot{M}_{O_2}$ values in the acid/Al fish (Fig. 1d).

Arterial pH decreased slightly, but significantly, from 7.80 to 7.74 over the first 4 h of acid/Al challenge (Fig. 3a). Although, as seen in Fig. 3a, there was a large drop in the average pHa between the prechallenge and final measurement (7.80 to 7.53), not all individuals experienced the same severity of acidosis prior to the terminal sample (Fig. 4a). Other than the slight increase in $Pa_{CO_2}$ in one fish within the first hour

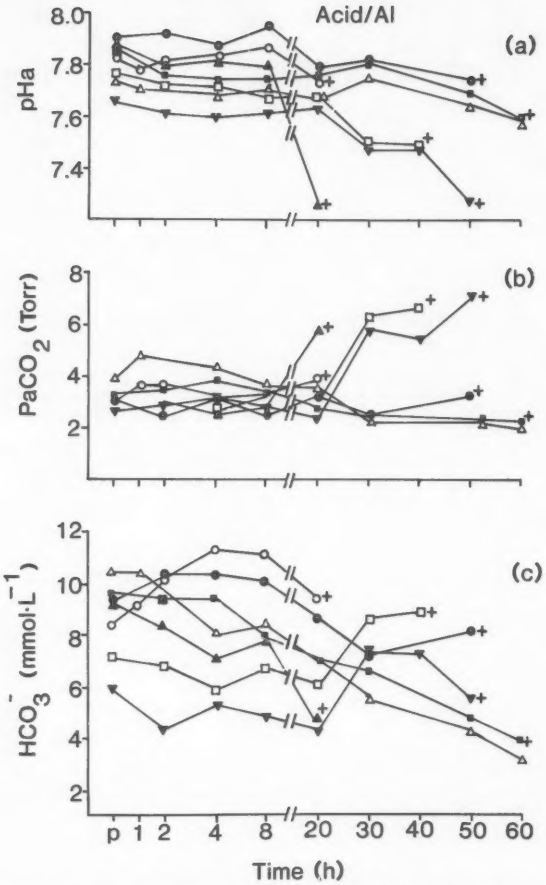


FIG. 4. Changes in the blood acid–base status of individual brook trout challenged with acid (pH 4.8) and Al (333 μg·L$^{-1}$) in flowing water at 11°C after acclimation to pH 6.5 ($Ca^{2+}$ = 400 μequiv·L$^{-1}$ in both acclimation and challenge water). (a) Arterial pH, pHa; (b) arterial $CO_2$ tension, $Pa_{CO_2}$ (calculated); (c) plasma bicarbonate concentration, $HCO_3^-$. The prechallenge values are indicated at "P." The plus signs indicate time of death. Only one fish survived to 100 h; therefore, only the first 60 h of exposure are plotted.

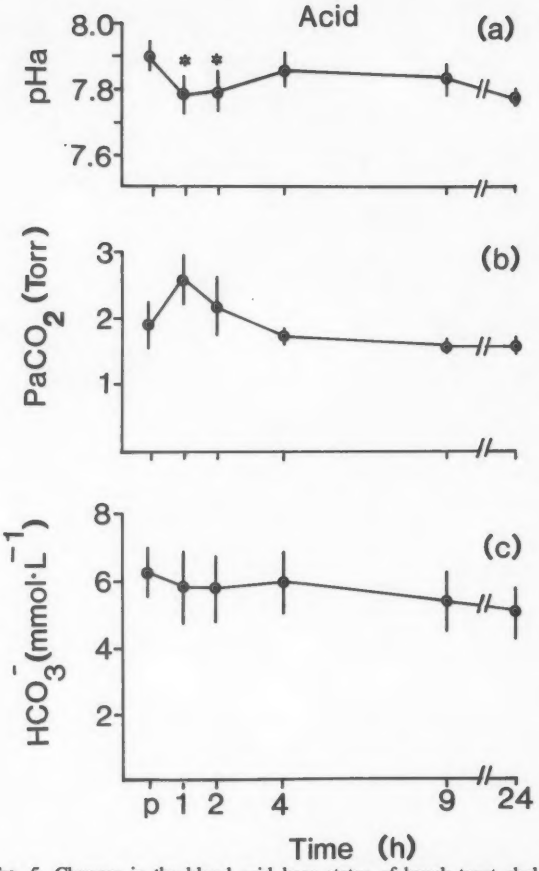


FIG. 5. Changes in the blood acid–base status of brook trout challenged with acid (pH 4.8) in flowing water at 11°C after acclimation to pH 6.5 ($Ca^{2+}$ = 400 μequiv·L$^{-1}$ in both acclimation and challenge water). (a) Arterial pH, pHa; (b) arterial $CO_2$ tension, $Pa_{CO_2}$ (measured); (c) plasma bicarbonate concentration, $HCO_3^-$. All points are means ±1 SEM ($n$ = 12). Other details as in legend to Fig. 2.

(Fig. 4b), there were no significant changes in either $Pa_{CO_2}$ or true plasma [$HCO_3^-$] during the experiment (Fig. 3b and 3c). Between 20 and 60 h there was a general trend toward a drop in [$HCO_3^-$] and an increase in $Pa_{CO_2}$ (i.e. combined metabolic and respiratory acidosis), but individual variability was substantial (Fig. 4b and 4c).

There was a mild acidosis evident within the first 2 h of exposure to acid in the absence of Al (Fig. 5a, 5b, and 5c), pHa falling from 7.89 to 7.78. Coincidental with the drop in pHa was an increase in $Pa_{CO_2}$ (measured) at 1 h (from 1.89 to 2.56 Torr, $n$ = 4). By the second hour of the acid challenge, $Pa_{CO_2}$ had returned to 2.17 Torr, which was not significantly different from the prechallenge value. No changes in plasma [$HCO_3^-$] were seen.

The changes in blood oxygen tension and oxygen content, expressed in terms of [Hb], were not dramatic in either study (Fig. 6, 7, and 8). In fact, rather than falling, $Pa_{O_2}$ tended to rise initially in both groups, although the changes were not significant; Fig. 7a demonstrates that at least half of the animals in series I (acid/Al) did experience a drop in $Pa_{O_2}$ before death. However, when averaged in with the other fish, there was no significant reduction in $Pa_{O_2}$ at the time of the terminal sample (Fig. 6a). $Pa_{O_2}$ of the fish challenged with acid alone was not different from the prechallenge mean throughout the experiment (Fig. 8a).

$Pv_{O_2}$ was measured only in the series I experiments (Fig. 6a). Although there appeared to be a mild reduction in $Pv_{O_2}$ over the course of 8 h (19.2 to 10.8 Torr), the changes were not significant.

There was a significant decrease in $Ca_{O_2}$, which fell from a prechallenge value of 5.2 to 2.6 mmol·L$^{-1}$ in the acid/Al group prior to death (Fig. 6c and 7c). This represents a 50% decrease in oxygen content. However, when expressed per unit of Hb, the content fell from 66.8 to 54.6 μmol·g Hb$^{-1}$, an 18% decrease, which was significant, nonetheless. A similar reduction in $Ca_{O_2}$ (millimoles per litre) was observed in the acid–alone group, yet the content per gram of Hb remained unchanged.

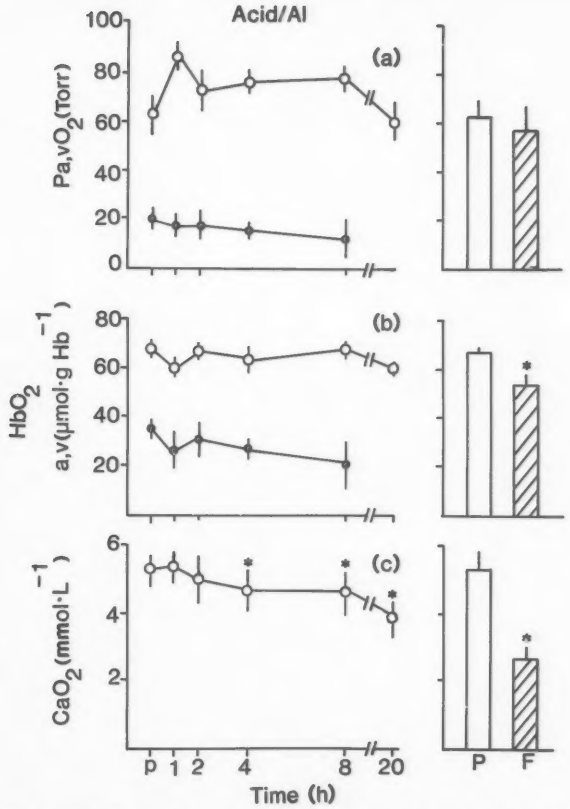


FIG. 6. Blood oxygen tension and content of brook trout challenged with acid (pH 4.8) and Al (333 $\mu g \cdot L^{-1}$) in flowing water at 11°C following acclimation to pH 6.5 $Ca^{2+}$ = 400 $\mu equiv \cdot L^{-1}$ in both acclimation and challenge water). (1) Arterial (open circles) and venous (closed circles) oxygen tension, $Pa,v_{O_2}$; (b) arterial (open circles) and venous (closed circles) hemoglobin oxygen concentration, $Hb_{O_2}$ a,v; (c) arterial blood oxygen content, $Ca_{O_2}$. All points are means ± 1 SEM ($n$ = 7). Other details as in legend to Fig. 1.

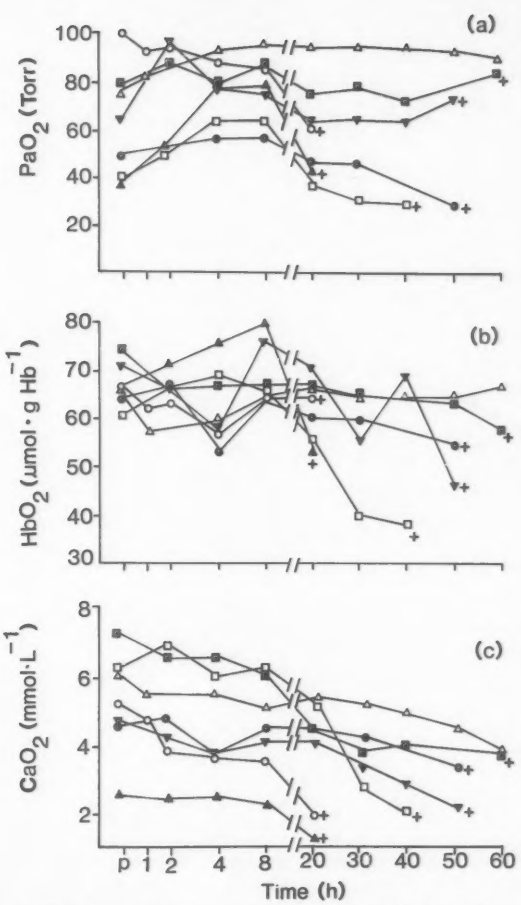


FIG. 7. Blood oxygen tension and content of individual brook trout challenged with acid (pH 4.8) and Al (333 $\mu g \cdot L^{-1}$) in flowing water at 11°C after acclimation to pH 6.5 ($Ca^{2+}$ = 400 $\mu equiv \cdot L^{-1}$ in both acclimation and challenge water). (a) Arterial oxygen tension, $Pa_{O_2}$; (b) arterial hemoglobin oxygen concentration, $Hb_{O_2}$; (c) arterial blood oxygen content, $Ca_{O_2}$. Other details as in legend to Fig. 4.

Almost every individual showed a decrease in MCHC prior to death during the acid/Al challenge, with the result that the final average MCHC was significantly less than the prechallenge value (0.216 vs. 0.260 $g \cdot mL^{-1}$, respectively; Fig. 9c). [Hb] and Hct were reduced due to sampling, but not by the same amount (Fig. 9a and 9b). In the acid-challenged fish, there was a 30% decrease in [Hb] and Hct due to sampling, but there was no significant reduction in MCHC (Fig. 10).

## Discussion

The initial increase in ventilation seen in this study in response to acid/Al supports the results of earlier investigators (Rosseland 1980; Neville 1985). However, the subsequent return to prechallenge values after 4 h and the general lack of a ventilatory increase prior to death were unexpected considering the respiratory disturbances reported by Wood et al. (1988b) under similar conditions. Even when considering individual fish, there was no apparent trend toward an increase in ventilation in response to the combined acid/Al stress, disregarding the initial burst of ventilatory activity. This was especially surprising, since all fish in the acid plus Al treatment exhibited visual signs of respiratory distress such as an increase in coughing frequency and mucus production. This finding illustrates the value of direct measurements of $\dot{V}_w$ and suggests that the stressed fish may have been unable to achieve increased net ventilatory flow because of metabolic limitations, increased gill resistance due to mucus, flow reversal due to coughing, or some combination of these factors. In the case of three fish, substantial increases in ventilation rate and stroke volume were noted 20–30 h before death; however, this was not the trend for most. Those fish showing the ventilatory increase were also the ones that experienced the most dramatic decrease in Hb oxygen content (micromoles per gram of Hb) and $Pa_{O_2}$ (Fig. 7).

The immediate increase in ventilation seen in all acid- and acid/Al-treated fish at 0.5 and 1 h of exposure may have been the result of an increase in the $P_{CO_2}$ of the water at the front end of the ventilation chamber. Although not directly measured in the acid/Al fish, the average $Pa_{CO_2}$, calculated from pHa and $HCO_3^-$, was slightly elevated (from 3 to 4 Torr) at 1 h. A

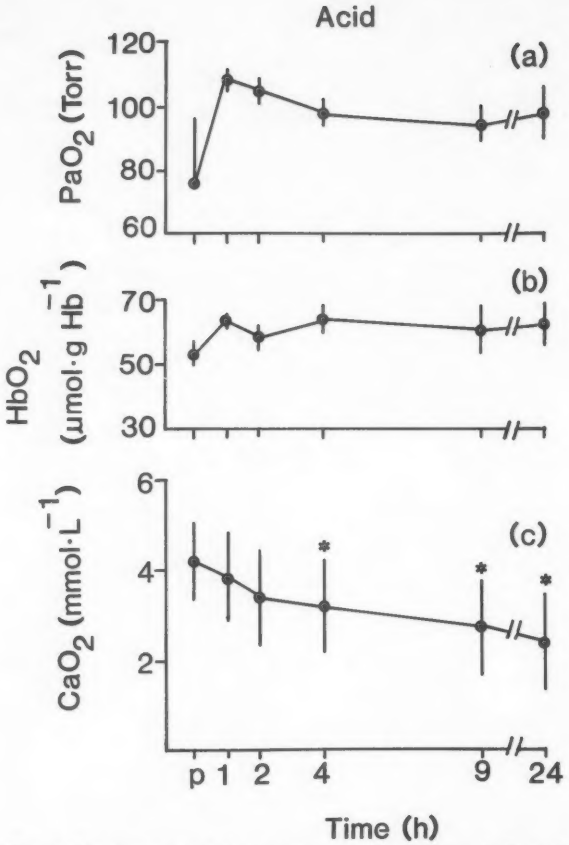


FIG. 8. Blood oxygen tension and content of brook trout challenged with acid (pH 4.8) in flowing water at 11°C after acclimation to pH 6.5 ($Ca^{2+}$ = 400 μequiv·L$^{-1}$ in both acclimation and challenge water). (a) Arterial oxygen tension, $Pa_{O_2}$; (b) arterial hemoglobin oxygen concentration, $Hb_{O_2}$; (c) arterial blood oxygen content, $Ca_{O_2}$. All points are means ±1 SEM ($n$ = 3). Other details as in legend to Fig. 2.

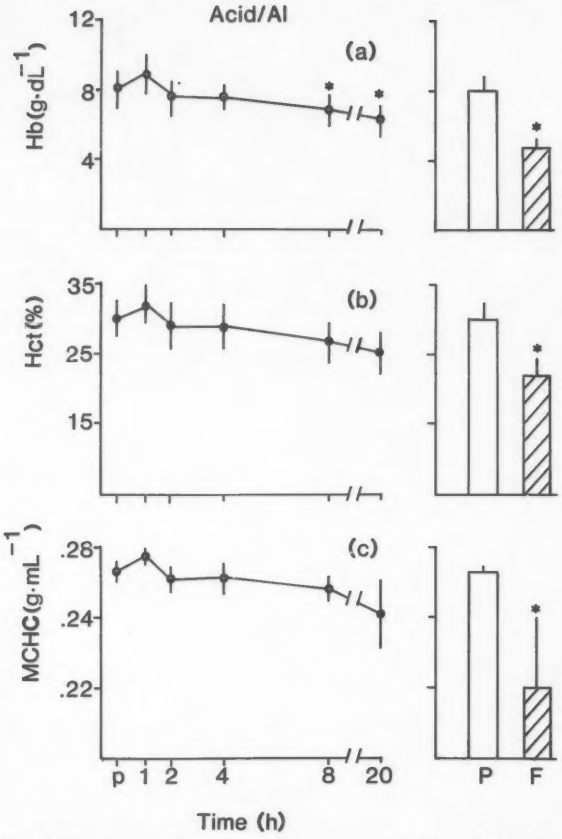


FIG. 9. Hematological responses of brook trout challenged with acid (pH 4.8) and Al (333 μg·L$^{-1}$) in flowing water at 11°C following acclimation to pH 6.5 ($Ca^{2+}$ = 400 μequiv·L$^{-1}$ in both acclimation and challenge water). (a) Hemoglobin concentration, Hb; (b) hematocrit, Hct; (c) mean cell hemoglobin concentration, MCHC. All points are means ±1 SEM ($n$ = 7). Other details as in legend to Fig. 1.

similar increase in $Pa_{CO_2}$ was directly measured in the acid-exposed fish. Neville (1979) found that an increase in inspired $P_{CO_2}$ from less than 1.5 to 2.2 Torr was enough to significantly raise ventilation. Similar findings were reported by Janssen and Randall (1975). During the initial hour of acid or acid/Al exposure of our experiments, pH = 4.8 water was unavoidably mixed with the preceding pH = 6.5 water in the inspired chamber of the ventilation box. The water had a carbonate alkalinity of 2.4 mg·L$^{-1}$ at pH = 6.5 which was reduced to less than 0.01 mg·L$^{-1}$ when acidified to pH = 4.8. The free $CO_2$ released during addition of the pH = 4.8 water may have raised the inspired $P_{CO_2}$ to a level sufficient to stimulate ventilation. The field studies reported by Rosseland (1980) and Muniz and Leivestad (1980) support the idea that initial ventilatory bursts are due to transient increases in water $P_{CO_2}$. In Rosseland's study there was an initial burst in ventilation that was followed by a decline. Therefore, the conditions reported in our study are not at all unlike the situation that one might expect during an episodic acid load in the natural environment.

Since hyperventilation persisted at least 9 h (and perhaps longer) at pH = 4.8, why did $\dot{V}_w$ return to control levels after only 4 h exposure to pH = 4.8 plus Al? We cannot rule out some sort of alleviating effect of Al on the stimulation of ventilation caused by external acidity. Neville (1985) noted that short-term increases (but not long-term increases) in $f_R$ and ventilatory "gape" were attenuated by the presence of Al at pH = 4.0. However, it seems more likely that mucus clogging, coughing reversals of flow, or energetic limitations caused by Al toxicity may have depressed $V_r$ and therefore $\dot{V}_w$ in our fish.

The initial acid–base disturbances were similar in both treatments; a slight decrease in pHa was observed within the first 2–4 h. The only major reduction in plasma pH occurred shortly before death in the acid/Al-treated fish. In some fish the respiratory component (increased $Pa_{CO_2}$) predominated, while in others the metabolic component (depressed $[HCO_3^-]$) was paramount. Because of this variability, terminal $[HCO_3^-]$ and $Pa_{CO_2}$ were not significantly different from the controls. In general, our results compare favorably with those of Wood et al. (1988c) who also noted the development of a severe acidosis shortly before death in Wyoming brook trout acclimated to the same conditions (pH = 6.5, $Ca^{2+}$ = 400 μequiv·L$^{-1}$) and exposed to the same acid/Al regime (pH = 4.8, $Ca^{2+}$ = 400 μequiv·L$^{-1}$, Al = 333 μg·L$^{-1}$) used in this study. In addition, these investigators reported a substantial increase in plasma lac-

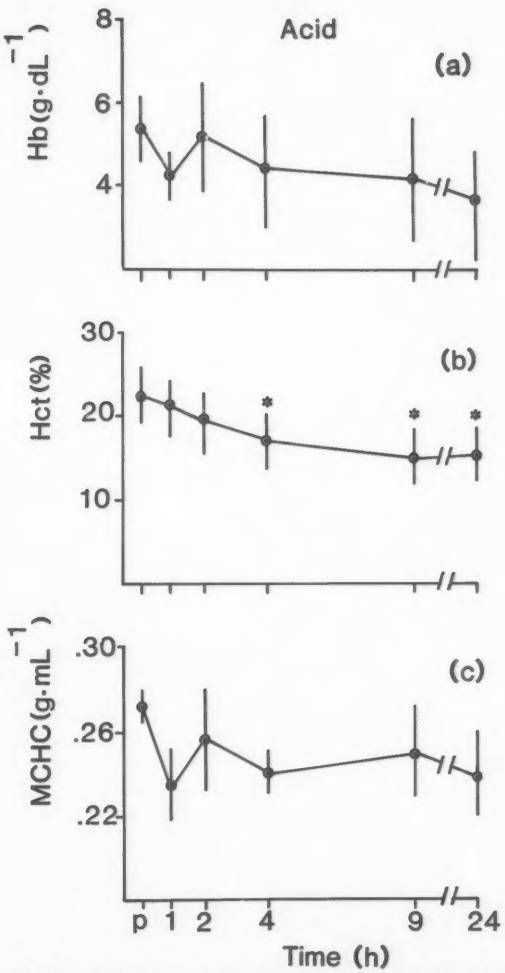


FIG. 10. Hematological responses of brook trout challenged with acid (pH 4.8) in flowing water at 11°C after acclimation to pH 6.5 ($Ca^{2+}$ = 400 μequiv·$L^{-1}$ in both acclimation and challenge water). (a) Hemoglobin concentration, Hb; (b) hematocrit, Hct; (c) mean cell hemoglobin concentration, MCHC. All points are means ±1 SEM ($n$ = 4 for Fig. 10a and 10c and $n$ = 8 for Fig. 10b). Other details as in legend to Fig. 2.

tate prior to death, which is indicative of metabolic acidosis. The two studies are also in agreement in showing severe reductions in MCHC during the acid/Al challenge, but not during the challenge to acid alone. This response is indicative of stress and probably reflects swelling of the red blood cells due to a combination of plasma dilution, acidosis, and catecholamine mobilization into the bloodstream, as previously discussed (Wood et al. 1988b, 1988c).

The effects of the acid-alone challenge upon blood oxygen tension were negligible, while the responses to acid/Al were rather variable. Initially, there appeared to be an increase in $Pa_{O_2}$ in both treatment groups, probably as a result of the increase in ventilation. While there were no effects of the acid alone on $Pa_{O_2}$ or Hb oxygen content (micromoles per gram of Hb), at least half of the fish in the acid/Al group exhibited a decrease in $Pa_{O_2}$ prior to death, and virtually all the fish showed a decrease in Hb oxygen content. Only the latter was statistically significant, and averaged about 20%. Based on in vivo and in vitro oxygen dissociation curves for brook trout blood (R. L. Walker, C. M. Wood, and C. E. Booth, unpubl. results), the Bohr effect associated with the reduction in plasma pH was more important than decreased $Pa_{O_2}$ in causing reduced Hb saturation. Reduced Hb oxygen content of this magnitude was unlikely the sole cause of death, but likely a contributing factor. It remains unclear why respiratory failure did not predominate, as seen in Ontario brook trout under identical conditions (Wood et al. 1988b).

Although the amount of oxygen carried per gram of Hb remained fairly constant, the total oxygen content decreased steadily during the experiment. The role of anemic hypoxemia cannot be ruled out as a factor affecting ventilation, since both [Hb] and Hct were reduced in both groups during the challenge periods, probably as the result of sampling (Fig. 9 and 10). Smith and Jones (1982) have demonstrated that an anemia sufficient to reduce $Ca_{O_2}$ content by 32% can cause a 1.6-fold increase in ventilation in trout. In this study the oxygen content was reduced by a similar amount. Therefore, the fact that ventilation remained elevated in the acid-alone group may be attributed to the hypoxemia associated with sampling, at least in part.

Hypoxemia was also evident in the acid/Al fish prior to death, yet there was no ventilatory response. A general lack of hyperventilation in this group, despite a significant reduction in $Ca_{O_2}$ and Hb saturation, suggests that Al is interfering with the mechanism controlling ventilatory response to hypoxemia. The mechanism of this interference remains to be elucidated.

## Acknowledgements

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# Effect of Long-Term Exposure to Acid, Aluminum, and Low Calcium on Adult Brook Trout (*Salvelinus fontinalis*). 1. Survival, Growth, Fecundity, and Progeny Survival

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Adult brook trout (*Salvelinus fontinalis*) were exposed to concentrations of acid, Al, and Ca representative of acidic and acid-sensitive surface waters. At low pH (4.42–5.03), survival and growth were reduced by elevated Al concentrations (486 μg/L) and low Ca concentrations (0.5 mg/L). Fecundity (number of eggs per female) was reduced by exposure to some treatment combinations, but this effect was mediated through reduced growth; number of eggs per unit body weight was not related to treatment. Viability of eggs from all parental exposures was high when incubated in neutral water. In spite of this high viability, eggs from parents exposed to low Ca concentrations showed greater mortality when incubated in the parental exposure conditions than did eggs from unexposed parents. Although the potential for such "carryover effects" cannot be discounted, we conclude that impairment of egg production is not a likely mechanism for loss of brook trout populations from acidic surface waters.

On a exposé des adultes d'omble de fontaine (*Salvelinus fontinalis*) à diverses concentrations d'acide, d'Al et de Ca semblables à celles présentes dans les eaux de surface acides et sensibles à l'acide. À un faible pH (4,42–5,03), les taux de survie et de croissance ont été abaissés par une concentration élevée d'Al (486 μg/L) et une faible teneur en Ca (0,5 mg/L). Le taux de fécondité (nombre d'oeufs par femelle) a été abaissé par l'exposition à certaines combinaisons expérimentales mais cette incidence était aussi le résultat d'un plus faible taux de croissance; le nombre d'oeufs par unité de poids corporel n'était pas lié au traitement. La viabilité des oeufs provenant de femelles exposées aux diverses conditions expérimentales était élevée quand ils étaient incubés dans un milieu neutre. Malgré cette viabilité élevée, les oeufs provenant de femelles exposées à une faible teneur en Ca ont montré un taux plus élevé de mortalité quand ils ont été incubés dans les mêmes conditions auxquelles les parents étaient exposés que les oeufs provenant de parents non exposés. Quoique le potentiel de tels « effets de report » ne puisse être ignoré, les auteurs formulent la conclusion que l'altération de la capacité de production d'oeufs n'est probablement pas responsable de la disparition des populations d'ombles de fontaine dans les eaux de surface acides.

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Acidification of surface waters and associated water chemistry changes have been implicated in losses of fish populations in Scandinavia (Jensen and Snekvik 1972; Rosseland et al. 1986), eastern Canada (Beamish and Harvey 1972; Beggs and Gunn 1986), and the northeastern United States (Haines and Baker 1986). Although episodes of acidic water and associated fish kills have been documented in some watersheds (Leivestad and Muniz 1976), field studies have suggested that at least some population losses may occur more gradually (Schofield 1976). In most cases, these gradual declines have been attributed to recruitment failure, leaving populations consisting of only older adult fish (Beamish 1974; Rosseland et al. 1980). Without successful annual recruitment, these populations slowly disappear.

In recent years, several mechanisms have been proposed to explain recruitment failure of fish populations in acidic waters, but most are encompassed by two hypotheses: (1) mortality of sensitive early life stages (Jensen and Snekvik 1972; Schofield 1976) or (2) failure of adult fish to produce viable eggs (Beamish and Harvey 1972; Beamish et al. 1975; Frenette and Dodson 1984). Although effects of acidification and associated water chemistry changes on egg production have been reported, they are difficult to document unequivocally in the field. Much of the field evidence that does suggest reproductive impairment comes from white sucker (*Catostomus commersoni*) populations in Ontario. Here, female white suckers have not shown the changes in plasma Ca concentration characteristic of vitellogenesis (Beamish et al. 1975; Lockhart and Lutz 1977), and resorption of eggs has also been reported (Beamish and Harvey 1972). In contrast, Gunn and Keller (1984) and Mills (1984) both found that acid-stressed populations of lake trout (*Salvelinus namaycush*) spawned normally, yet emergent fry were not found, suggesting mortality of early life stages. Although these data appear to conflict, there is no assurance, even within a single species, that the mechanism of recruitment failure is the same in all affected geographic regions or fish species. For

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example, many acidic lakes in the Sudbury region of Ontario have elevated concentrations of several heavy metals (Beamish 1974), which may elicit a different physiological response than would be expected in acidic waters receiving smaller inputs of heavy metals.

Brook trout (*Salvelinus fontinalis*) is one of the fish species most severely affected by acidification in the northeastern United States and Ontario (Haines and Baker 1986; Beggs and Gunn 1986). Two partial life cycle exposures of adult brook trout to acidic waters during vitellogenesis have been reported to date (Menendez 1976; Tam and Payson 1986). However, both of these studies used acid as the only experimental variable, and both were conducted using laboratory water with high Ca concentrations. Al, which is mobilized from minerals by acidic waters (Cronan and Schofield 1979; Driscoll et al. 1980) and is thought to be a major toxic factor in acidic surface waters (Baker 1982), was not included in these previous studies. Furthermore, high Ca concentrations, such as those used in both the Menendez and Tam and Payson studies, can ameliorate acid/Al stress (Brown 1981, 1982, 1983; Booth et al. 1988; Wood et al. 1988b). Ca is also important in the vitellogenic process (Whitehead et al. 1978), which may further invalidate the use of these studies for assessing potential effects on reproduction in soft acidic surface waters.

In this and the companion study (Mount et al. 1988), our objective was to determine for adult brook trout (1) the toxicological and physiological effects of long-term exposure to acid, Al, and low Ca, (2) the plausibility of reproductive failure as an explanation for observed recruitment failure in acidified surface waters, (3) the mechanisms by which reproductive failures might occur, and (4) the chemical conditions under which reproductive failures are likely to occur, if at all. Toward these ends, we conducted long-term exposures of reproducing adult brook trout to combinations of pH, Al, and Ca representative of chemical conditions in acidic surface waters. In this paper, we discuss the effect of such exposure on gross toxicological endpoints (survival, growth, and fecundity of adults and survival of progeny). The companion study (Mount et al. 1988) examined the effect of similar exposure on vitellogenesis and osmoregulatory status. Physiological and histological effects of exposure to acid, Al, and low Ca in adult brook trout are discussed in the accompanying series of papers on ionoregulation (Booth et al. 1988; Wood et al. 1988a, 1988c; McDonald and Milligan 1988), gas exchange (Wood et al. 1988b, 1988c), ventilation (Walker et al. 1988), and gill structure (Tietge et al. 1988).

## Methods

Results of two exposures (147 and 54 d) of adult brook trout to combinations of acid, Al, and Ca are presented in this paper. Both experiments were conducted at the University of Wyoming's Red Buttes Environmental Biology Laboratory, Laramie, WY.

### Experimental Design: 147-d Exposure

This experiment spanned 147 d (13 July to 7 December 1984) from early vitellogenesis to spawning and included 27 combinations of acid, Al, and low Ca. Combinations of pH and Al used were (nominal values) 0 μg Al/L at pH 6.5, 0, 18, 54, and 162 μg Al/L at pH 5.2, and 0, 54, 162, and 486 μg Al/L at pH 4.8. Each of these nine pH/Al combinations was tested at 0.5, 2, and 8 mg Ca/L, for a total of 27 combinations. Al concentrations were chosen to represent the range of concentrations common to surface waters of comparable pH in the Adirondack Mountain region of New York (Schofield 1982). Al concentration was not varied at pH 6.5 because of its low solubility at that pH and the consequently low concentrations found in neutral surface waters.

Fourteen 1.5-yr-old brook trout (mean starting weight = 108.7 g; standard error of the mean (SEM) = 2.2; Twin Buttes Trout Hatchery, Durango, CO) were exposed in each treatment combination; individual treatments were not replicated. Fish in all treatments were weighed before the experiment began and again on day 63; growth for each treatment was then expressed as percent change in mean body weight (mean weight on day 63 − mean weight before exposure)/(mean weight before exposure) × 100%. On day 83, three females from 12 treatments, representing a range of all three variables, were sacrificed to assess possible effects on ovarian development. Ovaries from these fish were weighed and then preserved in 10% neutral-buffered formalin for later dissection and egg counting. Since the removal of these fish was not random, we have used data from days 0–63 only for survival and growth analyses.

As spawning season approached, fish were checked weekly for reproductive maturity. Female fish were considered mature when eggs flowed freely from the ovipore with only gentle pressure on the abdomen. When mature, eggs were hand-spawned and fertilized using two or more male fish from the same treatment. To determine viability (defined as survival to hatch), we incubated a subset of 50 eggs from each treatment in neutral water (pH 6.5, 2 mg Ca/L). Sensitivity of the progeny to further exposure was determined by incubating another subset of 50 eggs in the inflowing water to the parental treatment. For comparison, similar subsets of eggs from control parents (pH 6.63, 8 mg Ca/L) were incubated simultaneously in the inflow to each parental treatment. Subsets of eggs used for progeny viability and sensitivity exposures consisted of equal numbers of eggs from each mature female fish; only samples from two or more females were used. Eggs not used for these studies were preserved in 10% neutral-buffered formalin for later counting and diameter measurement. Failure of the water system on day 147 forced termination of the adult exposure and the progeny sensitivity exposure (after 9 d). The viability determination continued through to hatch.

### Experimental Design: 54-d Exposure

This exposure lasted 54 d from 15 May to 22 July 1985. Twenty-five fish (mean starting weight = 164.1 g; SEM = 2.1; Cline's Trout Farm, Boulder, CO) were exposed to eight unreplicated combinations (nominal values) of pH (6.5 and 5.0), Al (0, 75, and 150 μg/L), and Ca (0.5 and 8 mg/L); as in the 147-d exposure, Al was not an experimental variable at pH 6.5. Each fish was uniquely finclipped to allow for individual identification and weighed at the beginning and end of the exposure. Growth for each treatment was expressed as the mean of the individual growth measurements for each fish surviving the exposure. Following this exposure, test fish were used for physiological experiments (see Wood et al. 1988a, 1988c; McDonald and Milligan 1988; Walker et al. 1988). After 16 d of exposure, mortality was as high as 28% in some treatment combinations, and nominal pH in all six acid treatments was raised from 5.0 to 5.2 on day 16 to slow mortality.

TABLE 1. Measured total Al, Ca, and Na concentrations on day 25 of the 147-d exposure, averaged by nominal concentration.

| Nominal concentration | Measured concentration | | | |
|---|---|---|---|---|
| | Mean ± SEM | Max. | Min. | *n* |
| Ca (mg/L) | | | | |
| 0.5 | 0.5 ± <0.1 | 0.6 | 0.4 | 8 |
| 2.0 | 1.6 ± <0.1 | 1.7 | 1.5 | 8 |
| 8.0 | 6.4 ± 0.2 | 7.0 | 5.7 | 9 |
| Total Al (μg/L) | | | | |
| 0 | 4 ± 2 | 8 | 2 | 3 |
| 18 | 20 ± 3 | 24 | 14 | 3 |
| 54 | 53 ± 3 | 61 | 43 | 6 |
| 162 | 138 ± 8 | 162 | 122 | 5 |
| 486 | 393 ± 5 | 399 | 382 | 3 |
| Na (mg/L) | 11.0 ± 0.1 | 12.1 | 9.5 | 25 |

### Test Water and Exposure System

Test water for both exposures was artificial soft water, reconstituted from deionized water (for a complete description of the water system, see Mount 1987). Well water was treated by reverse osmosis and separate-bed deionization to a final conductivity of 0.1–4 μS/cm. Na (1 mg/L) and Ca (0.5 mg/L) were added (as chlorides); pH was adjusted to desired levels by automatic pH controllers (Leeds and Northrup pH analyzer/controller, model 7083) using dilute acid ($H_2SO_4$) or base (NaOH for the 147-d exposure and KOH for the 54-d exposure). Function of the pH controllers was continuously monitored and integrated with the laboratory alarm system.

Test concentrations of Al and Ca were achieved by addition of $AlCl_3$ and $CaCl_2$ stock solutions to continuous-flow diluters. Test chambers were 340-L, round fiberglass tanks (Frigid Units, Inc.), each receiving moderate aeration. Volume additions per day were 2.2 for the 147-d exposure and 6.8 for the 54-d exposure. Temperature was varied from 9 to 14°C, according to the season. Photoperiod was adjusted every 2 wk to simulate the natural annual cycle for Denver, CO.

### Experimental Procedures

Upon arrival at our laboratory, fish were held in well water (hardness 250 mg $CaCO_3$/L, 52 mg Ca/L, pH 7.8) for a minimum of 2 wk, after which the water was gradually changed to artificial soft water (pH 6.5, 2 mg Ca/L) over 1 wk. All fish were held in this water for a minimum of 4 wk and then arbitrarily assigned to exposure chambers. To initiate exposure, we adjusted Ca concentrations to test levels and then reduced pH to desired levels over the course of several days (approximately 0.2–0.3 unit/d). Once desired pH was attained, Al delivery was started. The first day of Al addition was designated as day 0.

Fish in both exposures were fed trout chow at a rate of 1% wet body weight daily. During the first 81 d of the 147-d exposure, fish were fed sinking food (Rangens, Inc.), but this food tended to break apart and suspend in the water if uneaten. To avoid introducing this organic material, we changed the food to floating pellets (Purina No. 5106) on day 82. The floating pellets better retained their integrity in water, so the uneaten food could be removed more effectively. Floating pellets were used for the remainder of the 147-d exposure and during all of the 54-d exposure. Uneaten food was removed, and tanks were drained to half depth (to remove particles) daily in both experiments.

Survival, temperature, and delivery of stock solutions were monitored daily. During the 147-d exposure, pH of the inflowing waters was measured and adjusted daily (Sargent-Welch pH meter, model LS; Ross Orion combination electrode); pH of all exposure tanks was measured approximately weekly. In the 54-d exposure, pH was measured daily in every exposure tank.

### Analytical Chemistry

Water samples for chemical analysis were taken directly from the exposure tank. Cations ($Al^{3+}$, $Ca^{2+}$, and $Na^+$) were measured by atomic absorption spectroscopy (Perkin-Elmer model 2380) using flame or graphite furnace and standard methods (USEPA 1979). Anions ($Cl^-$ and $SO_4^{2-}$) were measured by anion chromatography (Dionex model 2110i; AS-40 column). For the 54-d exposure, Al was fractionated into total, monomeric (fast-reactive), and inorganic monomeric (dialysis/fast-reactive) after the method of LaZerte (1984).

During the 147-d exposure, Al and Ca concentrations were measured only once (day 25) to verify nominal concentrations. Al and Ca concentrations were close to nominal (Table 1), indicating that the diluter and toxicant delivery systems were of appropriate design and were operating properly. This, combined with daily monitoring of diluter function and stock solution delivery, suggests that in lieu of measured chemistry, nominal concentrations are a reasonable approximation of the actual exposure concentrations for Al and Ca during this exposure. As would be expected, measured pH in exposure tanks during the 147-d exposure deviated from nominal, particularly in treatments with high Al concentrations (Table 2). Because of its ability to complex hydroxide, Al can act as a buffer in acid solutions (Johannessen 1980), maintaining lower pH in waters with high concentrations. In addition, fish in high-Al exposures generally fed less than other fish, which would lower the input of ammonia and other metabolic byproducts that would raise pH. We accounted for these deviations by using measured pH in all data analyses, although this partial collinearity between pH and Al could potentially make it difficult to separate the independent effects of pH and Al.

During the 54-d exposure, weekly samples were taken for Al, Ca, Na, chloride, and sulfate (Table 3). Measured chemistry was used in all data analyses and presentations.

### Statistics

Survival data from the 147-d exposure and the progeny exposures were analyzed using stepwise logistic regression (BMDP, LR; Dixon and Brown 1985). Survival was assumed to fit a logistic distribution:

$$E(y) = \frac{\exp(B_0 + B_1X_1 + \ldots + B_nX_n)}{1 + \exp(B_0 + B_1X_1 + \ldots + B_nX_n)}$$

where $E(y)$ is the expected survival, $B_0$ to $B_n$ are the parameters of the regression model, and $X_1$ to $X_n$ are the independent variables. Stepwise linear regression (SPSS New Regression; Nie and Hull 1981) was used for other measured biological parameters. All dependent variables were regressed against both nontransformed and log-transformed chemistry values, except for acidity which was always expressed as pH. Our goal in the regression analyses was to define simple models that would identify the key variables relating to each measured response. For this reason, regression models were initially developed using only linear and quadratic main effects. Then a second

TABLE 2. Summary of the exposure matrix, survival, growth, egg production, and progeny exposure data from the 147-d exposure. Treatments began with 14 fish/treatment.

| Measured pH (units)[a] | Nominal Al (μg/L) | Nominal Ca (mg/L) | Adult survival (%) | Mean growth (%)[b] | Mean eggs per female (*n*)[c] | Progeny survival to hatch in control water (%) | 9-d progeny survival in parental treatments | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | Exposed parents (%) | Control parents (%) |
| 6.63 ± 0.06 | 0 | 8 | 93 | 51 | 811 ± 163 (6) | 92.0 | 100.0 | 96.6 |
| 6.64 ± 0.06 | 0 | 2 | 86 | 65 | 599 ± 245 (3) | — | 83.3 | 100.0 |
| 6.64 ± 0.06 | 0 | 0.5 | 100 | 54 | 386 ± 68 (5) | 78.1 | — | — |
| 5.57 ± 0.08 | 0 | 8 | 100 | 53 | 650 ± 137 (9) | 87.1 | 96.2 | 100.0 |
| 5.43 ± 0.09 | 0 | 2 | 93 | 38 | 710 ± 109 (6) | 91.5 | 77.4 | 100.0 |
| 5.60 ± 0.07 | 0 | 0.5 | 100 | 65 | 945 ± 191 (5) | 88.3 | 7.7 | 80.6 |
| 5.49 ± 0.08 | 18 | 8 | 100 | 65 | 849 ± 192 (8) | 83.9 | 100.0 | 100.0 |
| 5.41 ± 0.07 | 18 | 2 | 100 | 60 | 677 ± 163 (6) | 49.6 | 100.0 | 100.0 |
| 5.55 ± 0.06 | 18 | 0.5 | 100 | 53 | 659 ± 184 (6) | 70.5 | 30.8 | 93.3 |
| 5.55 ± 0.05 | 54 | 8 | 100 | 59 | 540 ± 124 (8) | 51.1 | 100.0 | 97.0 |
| 5.59 ± 0.07 | 54 | 2 | 100 | 21 | 331 ± 97 (7) | 77.6 | 96.7 | 96.8 |
| 5.59 ± 0.07 | 54 | 0.5 | 93 | 54 | 743 ± 109 (7) | 60.1 | 29.4 | 100.0 |
| 5.35 ± 0.06 | 162 | 8 | 93 | 27 | 585 ± 206 (6) | 68.2 | 98.1 | 100.0 |
| 5.46 ± 0.07 | 162 | 2 | 93 | 26 | 635 ± 111 (8) | 82.1 | 88.0 | 100.0 |
| 5.36 ± 0.09 | 162 | 0.5 | 86 | 6 | 486 ± 173 (9) | 61.9 | 76.0 | 100.0 |
| 5.01 ± 0.13 | 0 | 8 | 93 | 70 | 680 ± 166 (7) | 58.2 | 100.0 | 100.0 |
| 4.91 ± 0.12 | 0 | 2 | 100 | 64 | 836 ± 287 (5) | 94.6 | 98.0 | 97.7 |
| 4.91 ± 0.13 | 0 | 0.5 | 100 | 48 | 565 ± 196 (4) | — | — | — |
| 4.82 ± 0.12 | 54 | 8 | 92[d] | 47 | 197 ± 41 (4) | 62.0 | 100.0 | 96.7 |
| 4.82 ± 0.09 | 54 | 2 | 100 | 39 | 603 ± 95 (3) | — | — | — |
| 4.72 ± 0.09 | 54 | 0.5 | 93 | −9 | 494 ± 155 (5) | 87.9 | 46.2 | 84.4 |
| 4.83 ± 0.12 | 162 | 8 | 100 | 75 | 852 ± 140 (4) | 85.6 | 98.0 | 88.6 |
| 4.75 ± 0.12 | 162 | 2 | 100 | 44 | 524 ± 103 (8) | 74.5 | 94.2 | 100.0 |
| 4.61 ± 0.09 | 162 | 0.5 | 86 | 26 | 879 ± 173 (4) | — | — | — |
| 4.42 ± 0.06 | 486 | 8 | 50 | −34 | 243 ± 54 (5) | 82.4 | 96.2 | 96.9 |
| 4.45 ± 0.06 | 486 | 2 | 79 | −2 | 325 ± 99 (3) | — | — | — |
| 4.42 ± 0.06 | 486 | 0.5 | 36 | −36 | 242 ± 35 (4) | — | — | — |

[a]Mean pH (± SEM) calculated as the arithmetic mean of the mean pH for each week of the exposure.
[b]Growth calculated as the change in mean weight over days 0–63 divided by the mean starting weight for that treatment × 100%.
[c]Eggs per female (± SEM) is expressed as the mean for the treatment, including data from ovaries removed on day 83.
[d]Began with only 13 fish.

model was generated by allowing the algorithm to consider interaction terms. For logistic regressions, variables were included if they significantly improved the goodness of fit chi square. For linear regressions, the *F* statistic was used to determine significance of the regression ($p < 0.05$), and only variables that increased the adjusted $r^2$ of the model were considered for inclusion in the final model. When selecting the best model equation, we attempted to explain the most variance with the fewest variables. We included interaction terms only when they allowed for clearer interpretation of the data.

Because the 54-d exposure involved relatively few exposure combinations, and mean pH varied among treatments with the same nominal pH, we elected to use one-way analysis of variance for growth data. Significance of pairwise comparisons was determined by the Student–Newman–Keuls procedure.

## Results

### 147-d Exposure

In all but one case (a 2% difference), regressions using nontransformed chemistry data explained as much or more of the observed variance than did log-transformed data. Consequently, we elected to limit "best equations" to those using nontransformed data. With the exception of the regression for progeny sensitivity, we found that all interaction terms added little to the interpretation of the data and therefore excluded them from all but the progeny sensitivity model.

Survival was generally high in all exposure conditions; only at the highest Al concentrations was survival reduced below 85% (Table 2). Logistic regression gave nominal Al concentration alone as the best fit to the survival data, accounting for 64% of the total variance (Fig. 1). Mean body weight increased from day 0 to 63 in all but four exposure combinations: the three 486 μg Al/L treatments plus the pH 4.72, 0.5 mg Ca/L, 54 μg Al/L treatment (Table 2). Growth, expressed as percent change in mean body weight, was related to Al and Ca concentrations only (Fig. 1). Although the effect of Ca was significant, Al again accounted for the majority of the explained variance. Examining the data directly suggests that reduced Ca affects growth to the greatest extent at low pH and elevated Al concentrations.

There was a slight delay in reproductive maturation of fish in low pH exposures, but maturation in all treatments was spread over days 133–147. Eggs could not be stripped from females in several pH 4.8 treatments on day 133, when the first females became ripe in higher pH treatments. However, by day 147

TABLE 3. Summary of the mean measured chemical parameters for the 54-d exposure. Sample sizes appear in parentheses below each parameter name. Standard error of each mean is listed in parentheses below each value. Units are mg/L except where noted.

| Measured chemical parameters ($n$) | Treatments (nominal chemical parameters) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | pH 6.5<br>Ca 8.0<br>Al 0 | 6.5<br>0.5<br>0 | 5.2<br>8.0<br>0 | 5.2<br>0.5<br>0 | 5.2<br>8.0<br>75 | 5.2<br>0.5<br>75 | 5.2<br>8.0<br>150 | 5.2<br>0.5<br>150 |
| pH (units) | 6.35 | 6.48 | 5.19 | 5.25 | 5.12 | 4.95 | 5.13 | 5.07 |
| (55) | (0.03) | (0.03) | (0.05) | (0.06) | (0.05) | (0.05) | (0.05) | (0.06) |
| Ca | 6.8 | 0.6 | 6.7 | 0.6 | 6.8 | 0.5 | 6.8 | 0.6 |
| (5) | (0.1) | (0.1) | (0.2) | (0.1) | (0.2) | (0.1) | (0.2) | (0.1) |
| Total Al (μg/L) | 4 | 4 | 3 | 3 | 81 | 77 | 136 | 156 |
| (5) | (1) | (2) | (1) | (1) | (10) | (5) | (3) | (9) |
| Monomeric Al (μg/L) | 3 | 4 | 3 | 3 | 73 | 89[a] | 134 | 147 |
| (6) | (1) | (1) | (1) | (1) | (5) | (5) | (6) | (5) |
| Inorganic Al (μg/L) | 3 | 4 | 1 | 2 | 21 | 17 | 62 | 48 |
| (3) | (1) | (2) | (1) | (1) | (7) | (3) | (12) | (14) |
| Na | 5.1 | 4.9 | 5.2 | 5.0 | 5.1 | 5.2 | 5.1 | 5.1 |
| (5) | (1.6) | (1.5) | (1.7) | (1.6) | (1.7) | (1.6) | (1.7) | (1.6) |
| Cl | 15.9 | 3.3 | 15.5 | 3.5 | 15.6 | 3.7 | 16.7 | 4.0 |
| (3) | (0.1) | (0.1) | (1.0) | (0.1) | (1.0) | (0.2) | (1.2) | (0.1) |
| Sulfate | 5.9 | 4.2 | 10.2 | 14.3 | 10.2 | 10.1 | 14.7 | 10.4 |
| (3) | (4.4) | (3.0) | (5.4) | (5.2) | (5.4) | (5.1) | (5.2) | (5.2) |
| Temp. (°C) | 11.1 | 11.1 | 10.7 | 10.7 | 10.7 | 10.7 | 10.7 | 10.7 |
| (52) | (0.1) | (0.1) | (0.1) | (0.1) | (0.1) | (0.1) | (0.1) | (0.1) |

[a]Reactive Al greater than total Al is theoretically impossible. This mean is due to one rather high value that may have been caused by sample contamination or analytical error.

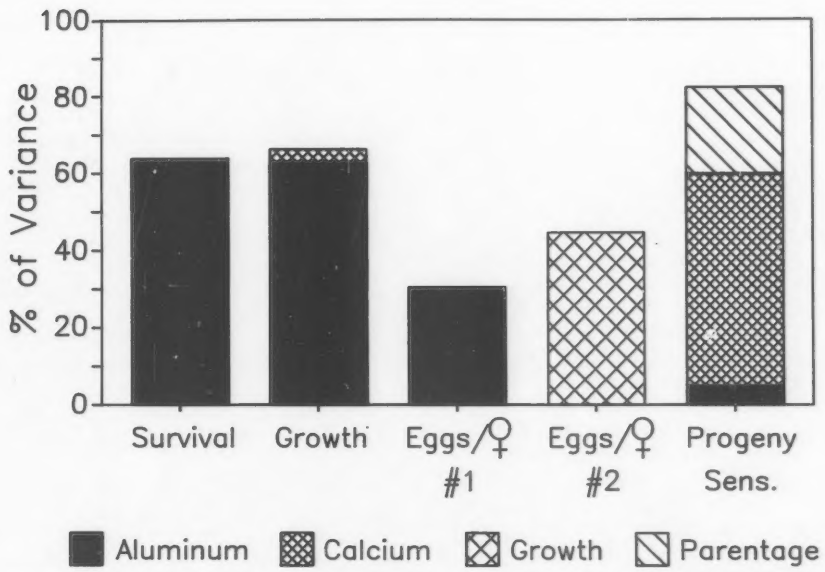


FIG. 1. Summary of multiple regression results from the 147-d exposure. Shaded areas represent the relative contribution of the addition of each variable to the model. Variance associated with interaction terms was split evenly between the variables. Models represented are (1) survival (logit survival = 3.25 − 0.0000130 Al$^2$; $r^2$ = 0.64); (2) growth (% change in weight = 47.88 − 0.159 Al + 1.92 Ca; $r^2$ = 0.67); (3) eggs/female No. 1, based on chemical variables only (eggs/female = 645 − 0.00155 Al; $r^2$ = 0.31); (4) eggs/female No. 2, including growth as an independent variable (eggs/female = 426.4 + 4.194 growth; $r^2$ = 0.45); (5) progeny sensitivity, with parentage = 1 for exposed parents and 0 for control parents (logit survival = 0.893 + 2.06 Ca − 0.222 Ca$^2$ − 3.26 parentage + 0.551 Ca × parentage + 0.0136 Al − 0.0000294 Al$^2$; $r^2$ = 0.82).

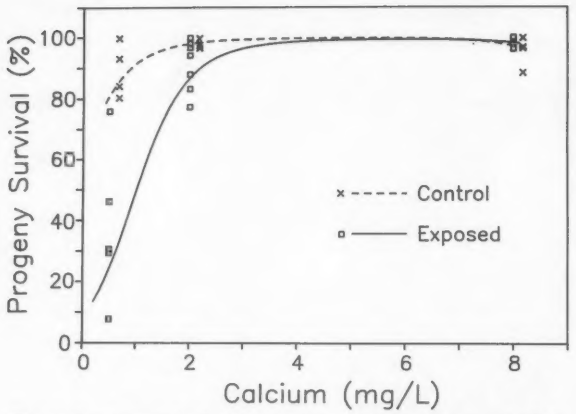


FIG. 2. Survival results from the progeny sensitivity exposure, including the fitted regression model. Broken line and ×'s represent eggs from control parents (pH 6.63, 8 mg Ca/L), and solid line and open squares represent eggs from exposed parents. The effects of Al, which accounted for 4% of the total variance, are not accounted for in this representation.

there was no clear relationship between pH and numbers of green/spent females. Most fish that had not matured by day 147 did contain nearly mature eggs.

Fecundity data were highly variable (Table 2). We expressed egg production as both eggs produced per female and eggs produced per 100 g of body weight, to account for differences in body size. Al concentration was inversely related to eggs per female, but explained only 27% of the variance. Although variability was high, the slope of this model was rather steep, predicting a 71% decrease in egg production over the range of 0–486 μg Al/L (688–197 eggs/female). In contrast, eggs/100 g body weight was not significantly related to pH, Al, or Ca concentrations. To increase sample sizes, we assumed that all maturing eggs in the ovaries sampled on day 83 would have matured and included those data in a new regression (Fig. 1). Once again, Al concentration was negatively related to eggs per female, but the improvement in $r^2$ was not large (0.31 compared with 0.27), and the predicted reduction over the range 0–486 μg Al/L remained high (57%). Finally, we included growth over days 0–63 as an independent variable, on the assumption that the effect of Al on fecundity might be mediated through growth (cf. nonsignificance of eggs/100 g regression). This regression included growth as the only independent variable (Fig. 1) and accounted for a greater percentage of the total variance ($r^2 = 0.45$) than did models based on treatment chemistry (Fig. 1). No chemical variable was selected as contributing significantly to this model. Diameter of ovulated eggs was not significantly related to treatment pH, Al, or Ca.

The developmental stage of the oocytes in ovaries removed on day 83 was estimated by dividing the total weight of the ovaries by the number of eggs in the ovaries (expressed as milligrams per egg). No relationships between egg weight and pH, Al, or Ca were significant. Both eggs per female and ovary weight showed relationships to Al and body weight similar to those recorded for fecundity; hence, these regressions were not considered further.

Viability (survival to hatch in neutral water) was high for eggs from all parental treatments (Table 2). Survival to hatch was above 50% for all but one parental exposure, and we believe that this exception was due to overripe eggs. Furthermore, there was no relationship between survival in control water and previous parental exposure.

TABLE 4. Survival and growth results from the 54-d exposure, including measured chemistry values. Mean pH for days 0–16 includes the period when all mortalities occurred and is prior to raising the nominal pH from 5.0 to 5.2. Mean pH for days 0–54 is the mean over the entire exposure. Growth is expressed as the mean of the individual growth measurements for each treatment.

| Mean pH, days 0–16 (units) | Mean pH, days 0–54 (units) | Measured Ca (mg/L) | Measured inorg. Al (μg/L) | Survival (%) | Growth (%) |
|---|---|---|---|---|---|
| 6.35[a] | 6.36 | 6.8 | 3 | 100 | 77 ± 4 |
| 6.52 | 6.50 | 0.6 | 4 | 100 | 67 ± 4 |
| 4.96 | 5.20 | 6.7 | 1 | 100 | 71 ± 4 |
| 5.03 | 5.26 | 0.6 | 2 | 100 | 53 ± 4[b] |
| 4.90 | 5.13 | 6.8 | 21 | 100 | 73 ± 5 |
| 4.73 | 4.99 | 0.5 | 17 | 72 | 11 ± 4[b] |
| 4.88 | 5.14 | 6.8 | 62 | 100 | 71 ± 4 |
| 4.78 | 5.09 | 0.6 | 48 | 84 | 28 ± 8[b,c] |

[a]Considered to be control conditions.

[b]Significantly different from control ($p = 0.05$).

[c]The variance of this treatment group was significantly greater than that of the other groups. To insure that this result was reliable, we used an approximate $t$-test for unequal sample sizes and unequal variances (Steel and Torrie 1960). The result was significant at $p < 0.001$ ($t = 5.52$).

In contrast with viability, sensitivity of eggs to continued exposure was affected by parental treatment. Survival of eggs from control parents (pH 6.63, 8 mg Ca/L) was >80% after 9 d of incubation in the inflow to all tested treatments, even at low pH and elevated Al (Table 2). However, eggs from exposed parents, when incubated in the inflow to the parental chamber, frequently showed greater mortality through 9 d than those from control parents. When survival of eggs from control and exposed parents was regressed against pH, Al, Ca, and parentage (using an indicator variable for parentage, with 0 = control parents and 1 = exposed parents), parentage, Ca, parentage × Ca interaction, and Al accounted for 82% of the variance (Fig. 1 and 2).

### 54-d Exposure

In this exposure, all mortalities were restricted to low pH, low Ca exposures (Table 4) and occurred before day 16 when the pH in low pH exposures was purposely raised approximately 0.2 pH unit. Although deaths were observed only in Al-containing exposures, mortality within low pH, low Ca treatments did not increase as Al concentration increased. However, the 0.5 mg Ca/L, 75 μg Al/L treatment, which had the greatest mortality (7 of 25 fish), also had the lowest mean pH over days 0–16.

Growth, expressed as mean percent change in body weight, ranged from 71 to 77% in all high Ca exposures, even at low pH and elevated Al. Growth was significantly reduced at all low pH, low Ca exposures (Table 4). These reductions were largest in treatments with reduced survival, but we observed no evidence of size-selective mortality. Growth was also lower in the pH 6.52, 0.6 mg Ca/L treatment than in any high Ca treatment, but this difference was not statistically significant.

## Discussion

This study is the first to simultaneously study the effects of exposure to low pH, elevated Al, and low Ca on adult brook

trout. Clearly, exposure to combinations of pH, Al, and Ca common to acidic surface waters can reduce both survival and growth of adult brook trout. Egg production can also be reduced, but only by reduced growth; egg production was not completely eliminated by any tested combination of pH, Al, and low Ca. Fish in all treatments produced viable eggs, even in exposures that reduced survival or growth. There was, however, an indication that exposure of adult fish to low Ca concentrations may decrease the resistance of eggs to continued exposure.

Since the toxic effects of low pH, low Ca, and elevated Al are highly interactive (Baker and Schofield 1982; Ingersoll et al. 1984), ascribing mortality to any one of the three variables can be difficult. However, mortality in the 54-d exposure appears to have been a consequence of low pH and low Ca concentration. Although the two treatments with reduced survival did contain Al, they also had the lowest mean pH values over days 0–16. Furthermore, toxicity of Al to brook trout has been shown to increase as pH increases (Baker and Schofield 1982); but raising the pH of these treatments on day 16 eliminated the mortality. If Al had been the cause of mortality in these treatments, raising the pH of the exposure should have exacerbated the mortality, rather than eliminating it.

For the 147-d exposure, regression analyses suggested that Al was largely responsible for the observed reductions in growth and survival (Fig. 1). Confounding these results, however, was the fact that treatments with the highest Al concentrations also had lower measured pH than did treatments with lower Al concentrations. To account for this, we created new regression models for survival and growth by eliminating Al as an independent variable and allowing the regression algorithms to consider only pH and Ca. For survival data, this procedure did give pH as the only significant variable, but $r^2$ was only 0.27, compared with 0.64 for the Al-based model. Likewise, pH contributed significantly to the new growth model, but again, $r^2$ was reduced from 0.67 (with Al) to 0.48. Based on these findings, we conclude that Al was indeed the major toxic factor in the 147-d exposure. This conclusion is consistent with physiological data collected from adult brook trout exposed to similar water chemistries (Booth et al. 1988; Wood et al. 1988b).

The observed sensitivities of adult brook trout to low pH, elevated Al, and low Ca are qualitatively similar to those documented for brook trout fry exposed in similar waters. Our results predict 75% survival of adult fish at 407 μg Al/L, while similar effects have been demonstrated at 111–333 μg Al/L for brook trout fry (Baker and Schofield 1982; Ingersoll 1986; Parkhurst 1987). In addition, adult brook trout were reasonably resistant to low pH stress alone, when Ca concentrations were >0.5 mg/L. Likewise, survival of brook trout fry is high at pH levels of 4.4 and above in the absence of Al (Baker and Schofield 1982; Ingersoll 1986). Only when Ca is reduced to 0.5 mg/L is survival of fry reduced (Ingersoll 1986). A Ca concentration of 0.5 mg/L is also stressful to brown trout (*Salmo trutta*) (Brown 1983).

In both exposures, treatments that reduced survival generally reduced growth as well. While food consumption was not directly quantified, fish in high-pH treatments usually consumed all food offered whereas fish in more stressful treatments were observed to leave much if not all food uneaten. Although there presumably are energetic costs of existence in acidic waters, reduced growth was undoubtedly due, at least in part, to refusal to eat. Indeed, exposure to low pH has been shown to affect responses to feeding stimuli in fathead minnows (*Pimephales promelas*) (Lemly and Smith 1987). While we cannot determine the cause and effect relationships involved, Cunningham and Shuter (1986) reported that starvation decreases whole-body ion concentrations in smallmouth bass (*Micropterus dolomieui*). In addition, histopathological studies on the gills of fish from the 147-d exposure showed structural responses indicative of ionoregulatory disturbance (Tiètge et al. 1988). Finally, Mount et al. (1988) showed a correlation between osmoregulatory status and growth in brook trout exposed to acid, Al, and low Ca. Since ionoregulatory distress is a common cause of death during acid/Al stress (Booth et al. 1988), the relationships among exposure, feeding, ionoregulation, survival, and growth may be very important.

Several effects of exposure to combinations of low pH, elevated Al, and low Ca on reproduction in adult brook trout were demonstrated, including reduced fecundity, delayed maturation, and reduced survival of progeny. Although eggs produced per female was reduced at higher Al concentrations, this effect was apparently mediated solely through reductions in growth. In fact, growth actually accounted for more of the variance in egg production than could be explained with chemical variables. Similarly, Tam and Payson (1986) showed reductions in eggs per female at low pH, but again, these effects were linked to reductions in growth; number of eggs per unit body mass was not different among treatments. Menendez (1976) found no effect on egg production at all. But most importantly, egg production was not completely eliminated at any exposure combination; complete elimination of egg production occurred only with death.

Laboratory studies on other species have shown effects of acid exposure on egg production. Fathead minnow spawning is particularly sensitive to pH stress, showing effects at pH levels well above the lethal threshold (Mount 1973). In flagfish (*Jordanella floridae*), low pH alone or combined with a mixture of metals has been shown to affect spawning (Craig and Baksi 1977; Hutchinson and Sprague 1986). But these effects could have been a result of behavioral modification, rather than reduced egg production per se; spawning pairs of flagfish transferred into stressful conditions immediately ceased spawning behavior even though they doubtless contained mature or nearly mature eggs (Hutchinson and Sprague 1986). This is particularly interesting, since white suckers in acidified lakes have been observed resorbing mature eggs (Beamish and Harvey 1972). Brook trout have shown the ability to discriminate between high- and low-pH water when selecting spawning sites (Johnson and Webster 1977), although Menendez (1976) found no effect of low pH on spawning behavior in brook trout. Eggs in our study were hand-stripped, so inhibition of spawning behavior would not have been recorded. Nevertheless, behavioral effects may be an important key to interspecific differences in reproductive effects of acid/Al stress.

Delayed maturation of brook trout in response to acid stress, as seen in the present study, has also been reported by Tam and Payson (1986), although Menendez (1976) found no such effect. While it appears that exposure to low pH can delay maturation, the consequences of such a response for natural populations are uncertain. Eggs of brook trout develop during the winter months, hatching as waters warm in the spring. Consequently, this hatching can be coincident with episodic increases in acidity and Al concentration associated with snowmelt, which would increase mortality of brook trout fry. But temporal variation of water chemistry during snowmelt is often quite high (Jeffries et al. 1979; Gunn and Keller 1984). Whether delayed

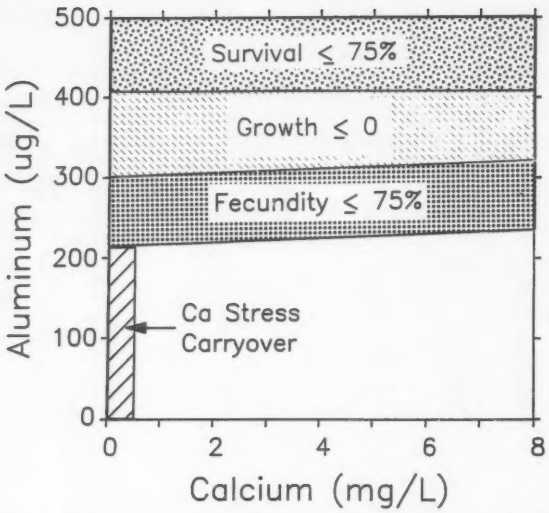


FIG. 3. Predicted responses of adult brook trout to combinations of Al and Ca at low pH (4.42–5.60). Predictions are based on regression models from the 147-d exposure and on the 54-d exposure.

maturation, and therefore delayed hatching, would make exposure of emergent fry to pulses of acidic waters any more or less likely is not clear, and may be purely a matter of chance.

Other studies have suggested the existence of "carryover effects" — effects of parental exposure to acidic waters on the viability of progeny, or the sensitivity of progeny to further exposure. Freeman and Sangalang (1985) found that eggs from Atlantic salmon (*Salmo salar*) in an acidic river were less viable than those of fish from a more neutral source. In the laboratory, Menendez (1976), Weiner et al. (1986), and Mount (1973), working with brook trout, rainbow trout (*Salmo gairdneri*), and fathead minnows, respectively, all demonstrated carryover effects. But the present study is the first to simultaneously examine the effects of Al and Ca, in addition to pH, as they relate to carryover effects. And despite the previous demonstration of carryover effects, two aspects of our findings are unique: (1) the importance of Ca and (2) the apparent mechanism of the effect.

In the regression model for progeny sensitivity, Ca was the only chemical variable to have a significant interaction with parentage, indicating that (low) Ca concentration was the cause of the observed sensitization. Furthermore, sensitization was consistently observed at low Ca concentrations, even at combinations of pH and Al that caused little or no stress in terms of survival or growth. Al was also included in the regression model, but there was no significant interaction with parentage. Hence, the toxic effects of Al were not specific to eggs from either exposed or control parents. Although pH was not significant in the progeny sensitivity regression, insufficient numbers of female fish were mature in the pH 6.64, 0.5 mg Ca/L treatment when the progeny sensitivity experiment was initiated. Therefore, the observed sensitization of progeny is known to be independent of pH only in the range of pH 4.42–5.60.

The mechanistic basis for previously demonstrated carryover effects appeared to be reduced viability of eggs from exposed parents. In some cases, this has been attributed to effects on male fish, leading to reduced fertility/viability (Weiner et al. 1986; Freeman et al. 1983). But this does not seem a plausible explanation in our study. Viability was high for eggs from all parental treatments; only when exposure was continued did differences between eggs from exposed and control parents appear. Since fertilization appears to have taken place normally, we believe that the carryover effect demonstrated here is due to effects on egg development, rather than effects on sperm or fertilization.

This carryover effect was the only demonstrated effect of acid, Al, and low Ca exposure on reproduction of brook trout which occurred in treatments which allowed for survival and growth of adult fish. Although scientifically intriguing, whether or not it bears significance in cases of environmental acidification is still unclear. Based on the present study, it appears that the carryover effect is independent of both low pH (4.42–5.60) and Al. However, we do not know whether this effect will take place in neutral waters of low Ca concentration or if some concurrent acid stress is necessary. If it can take place in neutral waters, then the carryover effect demonstrated here would have no direct relationship to population losses, as the stress would have been present before acidification. Moreover, Gunn (1986) asserted that in natural situations, brook trout eggs are incubated in alkaline groundwater upwellings. If this is indeed the case, then survival of progeny in the wild might be high regardless of parental exposure.

In addition, the influence of natural selection may mask the expression of a carryover effect. One would expect acidification of surface waters to place heavy selective pressure on natural fish populations, favoring genetic selection of eggs and fish better able to withstand these stresses. Such resistance has been demonstrated in yellow perch (*Perca flavescens*) from an acidified lake in the Adirondack Mountains (Schofield and Driscoll 1987). However, surface waters used in the perch study had higher Ca concentrations than those necessary to produce the carryover effect demonstrated here. The relative importance of genetic selection and carryover effects on sensitization/resistance of progeny has yet to be determined.

Overall, there is little doubt that low pH can be toxic to brook trout. But within the present studies, pH was not solely responsible for any of the observed reductions in survival, growth, fecundity, or survival of progeny. We concur, therefore, with the suggestion that adverse effects of acidic surface waters (pH $\geq$ 4.42) on adult brook trout are likely due, not to low pH as such, but to the interaction of low pH with concomitant elevated Al concentrations or low Ca concentrations (Baker 1982; Baker and Schofield 1982; Brown 1982). As a result, we can express the likely responses of adult brook trout at low pH (approximately 4.4–5.2) as a function of ambient Al and Ca concentrations (Fig. 3).

Based on the results of the 147-d exposure, 25% or greater mortality of adult brook trout would be expected at Al concentrations $\geq$407 μg/L (Fig. 3). In addition to this direct effect on survival, mean weight decreased in several treatments. Although these fish survived the experiment, fish that are losing weight cannot be expected to survive. For this reason, we also predict at least partial mortality at treatment combinations which cause weight losses, those in the range of 300–315 μg Al/L (Fig. 3). Finally, results from the 54-d exposure show that, at low pH, Ca concentrations $\leq$0.5 mg/L can reduce both survival and growth of adult brook trout. While no predictive model was generated for these data, this finding, combined with reductions in progeny survival, lead us to believe that combinations of low pH and $\leq$0.5 mg Ca/L also represent considerable stress for adult brook trout (Fig. 3). Bear in mind that these analyses are

not intended to establish threshold effect levels, but rather to identify areas at which substantial adverse effects are likely.

As stated in the introduction, one of the primary objectives of this study was to determine the plausibility of reproductive failure as an explanation for recruitment failure in acidic surface waters. No evidence was found to suggest that exposure to reasonable combinations of acid, Al, and low Ca can completely eliminate egg production, short of adult mortality. Fecundity can be reduced (via reduced growth) by combinations of Al and low Ca that allow survival of adult fish (Fig. 3), but only up to about 35%. The exact consequences of such a reduction to field populations will, of course, depend on the population dynamics of individual populations. But modeling of brook trout population responses to acidification suggests that reduced fecundity of female fish is less important to sustaining a population than are equivalent reductions in survival of yearling fish, and much less important than reduced survival of adult fish (Van Winkle et al. 1986).

It appears, then, that reproductive failure is not a likely explanation for recruitment failure in brook trout populations under acidification stress. Observed reductions in fecundity were relatively small and occurred only in combinations of acid, Al, and Ca that also interfered with survival and/or growth of adult fish. There was some suggestion that adult fish living in acidic, low Ca waters may have progeny that are more sensitive to continued exposure to those waters. Unfortunately, in natural populations, such an effect would be virtually indistinguishable from direct mortality of early life stages. Moreover, incubation of brook trout eggs in alkaline groundwater upwellings might prevent expression of a carryover effect. We suggest, therefore, that the mechanism of brook trout population loss in acidic surface waters is direct mortality of sensitive life stages, rather than effects on egg production.

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# Effect of Long-Term Exposure to Acid, Aluminum, and Low Calcium on Adult Brook Trout (*Salvelinus fontinalis*). 2. Vitellogenesis and Osmoregulation

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Adult brook trout (*Salvelinus fontinalis*) were exposed for 193 d (previtellogenesis to spawning) to six combinations of acid, Al, and low Ca. Survival and growth were reduced by low pH combined with low Ca concentrations. After 41 d of exposure, fish in all low pH exposures showed depressed plasma osmolality and Na concentrations, but by day 97 this apparent osmoregulatory stress was compensated for in all but the most severe treatment (pH 4.97, 47 μg inorganic Al/L, 0.5 mg Ca/L). At the observed peak of yolking (day 147), fish exposed to this treatment also had mean concentrations of plasma estradiol, vitellogenin, and Ca of only half control values. Fecundity (eggs per female) was significantly reduced as well, but this reduction was due in part to decreased growth. Despite these abnormalities in ionoregulatory and reproductive physiology, fish in all treatment conditions produced mature eggs. Among fish in stressful conditions, individual variation in growth and physiological parameters appeared to be correlated with osmoregulatory status. We hypothesize that the suite of physiological disturbances observed are linked to osmoregulatory impairment.

Pendant 193 d (de la prévitellogenèse au frai), on a exposé des adultes d'omble arctique (*Salvelinus fontinalis*) à six combinaisons d'acide, d'Al et de faibles teneurs en Ca. On a noté une baisse des taux de survie et de croissance chez les ombles exposés à un faible pH et à de faibles concentrations de Ca. Après 41 d d'exposition, l'osmolalité et les teneurs en Na du plasma étaient moindres chez les poissons exposés à un faible pH, mais dès le 97e jour, ce stress osmorégulatoire apparent avait été compensé chez tous les poissons sauf ceux exposés aux conditions les plus extrêmes (pH 4,97, 47 μg/L d'Al inorganique, 0,5 mg/L de Ca). Au pic observé de la vitellogenèse (147e jour), les poissons exposés à ces conditions montraient aussi des concentrations moyennes d'estradiol, de vitellogenine et de Ca plasmatiques qui ne dépassaient pas 50 % des valeurs témoins. Le taux de fécondité (nombre d'oeufs par femelle) a aussi été nettement abaissé mais cette réduction était en partie le résultat d'une croissance moindre. Malgré ces anormalités de la physiologie de l'ionorégulation et de la reproduction, les poissons exposés à toutes les conditions expérimentales ont produit des oeufs matures. Parmi les poissons exposés à un stress, la variation individuelle des paramètres physiologiques et de croissance semblait être corrélée à l'état osmorégulatoire. Les auteurs formulent l'hypothèse que l'enfilade de perturbations physiologiques observées est liées à l'altération de la capacité osmorégulatoire.

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Reproductive impairment in female fish has been hypothesized as a mechanism for recruitment failure in acidified surface waters, and there is evidence from both the laboratory (Ruby et al. 1977; Mount 1973; Hutchinson and Sprague 1986) and the field (Beamish and Harvey 1972; Beamish et al. 1975; Frenette and Dodson 1984) that reproductive failures are possible in certain species and watersheds. In particular, low pH exposure has been shown to inhibit oocyte yolking and development in flagfish (*Jordanella floridae*) and brook trout (*Salvelinus fontinalis*) (Ruby et al. 1977; Frenette and Dodson 1984).

In teleost fishes, oocyte yolking is a complex set of endocrine and anabolic processes (cf. Ng and Idler 1983; Ho 1987). Briefly, environmental cues (e.g. photoperiod, temperature) stimulate the release of gonadotropin from the pituitary into the bloodstream. At the ovary, gonadotropin induces synthesis and release of estradiol, which travels via the blood to the liver, where it in turn stimulates the synthesis of the lipoprotein vitellogenin, sometimes called "yolk-precursor protein." After synthesis, vitellogenin is secreted into the blood and transported to the ovary, where it is selectively incorporated into the developing oocytes. Vitellogenin is the source for most of the exogenous yolk in mature eggs.

Vitellogenin is a large protein, which is highly phosphorylated in a stoichiometric fashion. Since it is the only source (in relative terms) of protein-bound phosphate in fish plasma, vitellogenin can be easily quantified by a phosphoprotein–phosphate assay (Ng and Idler 1983). Also quantitatively bound to vitellogenin are calcium ions, which are important for keeping vitellogenin in solution (Whitehead et al. 1978). Because of the physiological relationships between estradiol, vitellogenin, and Ca, characteristic changes in their plasma concentrations occur seasonally in female salmonids. All three are present in relatively low concentrations from postspawning to the beginning

of exogenous or secondary yolking, at which time plasma estradiol (under gonadotropin regulation) begins to rise, peaking just before spawning. In response, plasma concentrations of both vitellogenin and Ca also rise to a peak around ovulation and then return to baseline levels following spawning.

Field evidence from white sucker (*Catostomus commersoni*) populations in the Sudbury region of Ontario suggests that exposure to low pH and associated water chemistry changes (e.g. increased metal concentrations) may indeed affect vitellogenesis (Beamish et al. 1975; Lockhart and Lutz 1977). These studies reported that during vitellogenesis, female fish in an acidic lake did not show the characteristic increases in serum Ca, retaining Ca concentrations equal to those of male fish. Meanwhile, females inhabiting nearby neutral lakes underwent normal changes in serum Ca concentration. Moreover, abnormalities in Ca metabolism were associated with populations of suckers failing to spawn successfully.

As discussed in the preceding paper (Mount et al. 1988), brook trout are perhaps the most widely affected of all fish species in the acid-sensitive watersheds of Ontario and the northeastern United States (Beggs and Gunn 1986; Haines and Baker 1986). In addition, Frenette and Dodson (1984) observed some inhibition of oocyte maturation in an acid-stressed brook trout population. Both Tam and Payson (1986) and Menendez (1976) have studied the effect of long-term exposure to low pH on brook trout survival, growth, and fecundity in the laboratory. Tam et al. (1986a) also studied steroid hormones, vitellogenin, and metabolic parameters in acid-stressed brook trout. But all of these laboratory experiments focused on acid as the only experimental variable and did not consider Al. Furthermore, all were conducted in laboratory water with higher Ca concentrations than would be expected in acid-sensitive surface waters (Brakke et al. 1988). Al is considered to be a major toxic factor in many acidic surface waters (Baker and Schofield 1982), while Ca is known to ameliorate many adverse effects of acid and Al exposure (Brown 1981, 1983; Ingersoll 1986).

The preceding paper (Mount et al. 1988) discussed the effect of various acid, Al, and Ca concentrations on the survival, growth, and fecundity of adult brook trout during long-term exposure in the laboratory. The present study explored the effects of similar exposures on vitellogenesis and osmoregulation and their relation to survival, growth, and fecundity.

## Materials and Methods

### Test Fish and Holding Conditions

Five hundred approximately 18-mo-old brook trout (Cline's Trout Farm, Boulder, CO) were held in our laboratory well water (alkalinity = 250 mg $CaCO_3$/L, 52 mg Ca/L) for 1 wk. Culture water was then changed to artificial soft water (pH 6.5, 0.5 mg Ca/L; see below); acclimation to this soft water continued for 5 wk before initiation of the experiment. From the original 500 fish, we selected 96 fish that appeared to be female on the basis of secondary sex characteristics for use in the experiment and arbitrarily assigned 16 fish to each of the six exposure tanks (mean weight = 164.1 g; SEM = 2.1). Eighteen of these 96 fish were later found to be male, with one to five present in each exposure tank.

Throughout holding and exposure, fish were fed 1% of body weight daily with floating trout chow (Purina No. 5106); uneaten food was removed and tanks were cleaned daily. Photoperiod was altered every other week to simulate the natural annual cycle for Denver, CO.

### Experimental Design

Six combinations of acid, Al, and Ca concentrations were tested (Table 1). For convenience, these treatments will be referred to as (1) neutral, high Ca (pH 6.40, 7.5 mg Ca/L), (2) neutral, low Ca (pH 6.48, 0.5 mg Ca/L), (3) acidic, high Ca (pH 5.04, 7.4 mg Ca/L), (4) acidic, high Ca + Al (pH 5.01, 7.4 mg Ca/L, 172 μg monomeric Al/L), (5) acidic, low Ca (pH 5.04, 0.5 mg Ca/L), and (6) acidic, low Ca + Al (pH 4.97, 0.5 mg Ca/L, 169 μg monomeric Al/L). The neutral, high Ca treatment (1) was chosen as a control treatment, as these conditions should represent the most favorable treatment combination and should therefore represent a relatively unimpaired response. Al was not an experimental variable at neutral pH because of its low solubility at neutral pH and consequent low concentrations in neutral surface waters.

On 22 May 1985, Ca concentrations were changed to experimental levels, and over the next 3 d, pH in the four acidic treatments (3–6) was lowered to 4.8. Al addition began on 31 May 1985, and this was designated as day 0. Exposure continued for 193 d (31 May 1985 to 10 December 1985), encompassing the period from previtellogenesis to spawning. Because

TABLE 1. Measured chemical and physical parameters averaged over the 193-d exposure period (mean ± SEM; units are mg/L except where noted). Sample size (*n*) appears in parentheses after each parameter.

| | Treatment | | | | | |
|---|---|---|---|---|---|---|
| Parameter (*n*) | (1) Neutral, high Ca | (2) Neutral, low Ca | (3) Acidic, high Ca | (4) Acidic, high Ca + Al | (5) Acidic, low Ca | (6) Acidic, low Ca + Al |
| pH (units) (192) | 6.40 ± 0.02 | 6.48 ± 0.02 | 5.04 ± 0.02 | 5.01 ± 0.02 | 5.04 ± 0.02 | 4.97 ± 0.02 |
| Ca (22) | 7.5 ± 0.1 | 0.5 ± 0.1 | 7.4 ± 0.1 | 7.4 ± 0.1 | 0.5 ± 0.1 | 0.5 ± 0.1 |
| Monomeric Al (μg/L) (21) | 4 ± 1 | 5 ± 1 | 6 ± 1 | 172 ± 33 | 5 ± 1 | 169 ± 34 |
| Inorganic Al (μg/L) (18) | 3 ± 1 | 2 ± 1 | 3 ± 1 | 81 ± 7 | 3 ± 1 | 47 ± 7 |
| Na (22) | 2.4 ± 0.6 | 2.3 ± 0.6 | 2.3 ± 0.6 | 2.3 ± 0.6 | 2.4 ± 0.6 | 2.3 ± 0.6 |
| Mg (μg/L) (8) | 16 ± 4 | 18 ± 4 | 15 ± 3 | 13 ± 3 | 21 ± 1 | 5 ± 3 |
| K (16) | 4.4 ± 1.1 | 4.4 ± 1.0 | 4.5 ± 1.1 | 4.1 ± 1.1 | 4.4 ± 1.1 | 4.5 ± 1.1 |
| Cl (20) | 15.8 ± 0.3 | 2.8 ± 0.1 | 15.5 ± 0.2 | 16.1 ± 0.3 | 2.8 ± 0.1 | 3.3 ± 0.1 |
| Sulfate (19) | 1.3 ± 0.8 | 1.3 ± 0.8 | 10.0 ± 1.8 | 8.9 ± 1.6 | 10.3 ± 1.8 | 9.2 ± 1.6 |
| Nitrate (μg/L) (14) | 143 ± 42 | 178 ± 65 | 136 ± 41 | 130 ± 39 | 125 ± 41 | 124 ± 37 |
| F (μg/L) (16) | 26 ± 4 | 23 ± 6 | 29 ± 7 | 24 ± 4 | 16 ± 3 | 26 ± 5 |
| Conductivity (μS/cm) (48) | 82 ± 3 | 35 ± 2 | 86 ± 3 | 87 ± 3 | 41 ± 3 | 41 ± 3 |
| Temperature (°C) (189) | 11.1 ± 0.1 | 11.1 ± 0.1 | 10.8 ± 0.1 | 10.8 ± 0.1 | 10.8 ± 0.1 | 10.8 ± 0.1 |

surviving fish were needed for physiological sampling, nominal pH in the acidic treatments (3–6) was raised from 4.8 to 5.0 on day 16 to slow the initial mortality. On day 80, an exposure system malfunction caused the dissolved oxygen in the neutral, low Ca treatment (2) to drop, and 6 of the 16 fish were lost. On day 148, this treatment (2) was accidentally contaminated with disinfectant, resulting in 100% mortality; hence, data from the terminal sampling are missing for this treatment.

### Test Water Preparation

Test water was artificial soft water, reconstituted from reverse-osmosis/deionized water (product water conductivity <0.1–4.0 μS/cm; for a complete description of the water system, see Mount 1987). To this water, 1.0 mg Na/L and 0.5 mg Ca/L were added (as chlorides) and then adjusted to pH 6.5 with an automatic pH controller (Leeds and Northrup model 7083) adding dilute KOH or $H_2SO_4$. This water was split and delivered to individual headtanks, one per test pH, where pH was left at 6.5 or lowered to 4.8, again using a pH controller and dilute $H_2SO_4$. These pH-adjusted waters were supplied to continuous-flow serial diluters, where desired Al and Ca concentrations (from chlorides) were maintained by addition of stock solutions from Mariotte bottles; delivery of these stock solutions was checked daily. Exposure chambers were 340-L, round fiberglass tanks (Frigid Units, Inc.) which were moderately aerated. Initially, each tank received test water at 1.35 L/min (5.7 volume additions/day), but due to growth of the fish, flow was increased to 1.8 L/min (7.6 volume additions/day) on day 90 of the exposure.

### Water Chemistry

During the experiment, pH (Sargent-Welch model LS, Orion Ross combination electrode) and temperature in the exposure tanks were recorded daily. Dissolved oxygen (Yellow Springs Instruments, Inc., model 54A) and ammonia (Orion Research Ionoanalyzer model 407A) were measured periodically and were maintained at >73% saturation (except for the deviation noted above) and <0.26 mg/L, respectively.

Water samples were taken weekly from all tanks and analyzed for $Al^{3+}$, $Ca^{2+}$, $Na^+$, $K^+$, $Mg^{2+}$, $Cl^-$, $SO_4^{2-}$, $NO_3^-$, and $F^-$, although not every parameter was measured at each sampling. Cations were analyzed using atomic absorption spectroscopy (AAS; Perkin-Elmer model 2380) and standard methods (USEPA 1979); flame emission was used for all cations except $Al^{3+}$ which was measured by graphite furnace. Al species were separated into total monomeric (fast-reactive) and inorganic monomeric (dialysis/fast-reactive) fractions after the method of LaZerte (1984). Anions were estimated by anion chromatography (Dionex model 2110i, No. AS-40 column).

Measured water chemistry values were generally close to nominal (Table 1). In the two treatments containing added Al (4 and 6), inorganic monomeric Al averaged 28 and 47% of the total monomeric Al. $Cl^-$ concentrations and conductivity varied across treatments depending on the $Ca^{2+}$ and $Al^{3+}$ concentrations, since these ions were added as chloride salts. $F^-$ concentrations were low enough (19–29 μg/L) to have negligible effect on the toxicity of Al (Parkhurst 1987).

### Blood Sampling

Blood samples were taken from all fish on four occasions: days 41, 97, 147, and at the termination of the exposure on day 193. Fish were anesthetized with MS-222 in tank water which was titrated to the exposure pH with KOH, and then a 500-μL blood sample was taken by puncture of the caudal blood vessels with a 21-gauge needle and a heparinized syringe. During the first sampling, each fish was also given a distinctive finclip to allow for individual identification at later samplings. Blood samples were placed on ice and processed within 30 min. Hematocrit was determined on a 50-μL aliquot of the sample; the remainder was centrifuged (5 min at 1000 × *g*) to separate cellular and plasma components. Plasma osmolality was measured by vapor pressure osmometry (Wescor model 5100 C). Two 20-μL plasma samples were each diluted with 4 mL of distilled water for Ca and Na determinations (by AAS). An additional 20-μL sample was diluted with 1 mL of phosphate-buffered saline with gelatin (PBS-gel; 0.1 M NaCl, 0.05 M $PO_4$, 0.1% gelatin, 0.01% thimerasol, pH 7.5) for estradiol radioimmunoassay. All dilutions (for ion and estradiol analyses) and the remainder of the plasma (for phosphoprotein-phosphate assays) were frozen and stored at −70°C until assayed.

### Spawning and Termination of the Experiment

All female fish were anesthetized, weighed, and manually stripped of mature eggs on three occasions, days 175, 184, and 193. Eggs were preserved in 10% neutral-buffered formalin for later counting and diameter measurement. Following the final spawning, blood samples were taken, and then all fish were sacrificed. The body cavity was opened, all coelomic eggs were removed and preserved for counting, and then the ovaries were removed and preserved in neutral-buffered formalin. At a later date, each ovary was blotted dry, weighed, and then qualitatively graded on a scale of 1–3, based on the number of large, yolky eggs remaining within the ovary (1 = <10, 2 = 10–50, 3 = >50). Gonadosomatic index (GSI) was calculated as (ovary weight/body weight) × 100%. Diameters of stripped eggs were measured to the nearest 0.1 mm on a subsample of 25 eggs, using a dissecting microscope at 20×.

### Estradiol-17β Radioimmunoassay

A specific radioimmunoassay for estradiol-17β was developed and validated for brook trout plasma. Depending on the anticipated estradiol concentration, 20–100 μL of the diluted plasma sample (above) was extracted with 3 mL of 1:1 benzene/hexane (by volume) for 30 s on a vortex mixer. After extraction, tubes were cooled at −20°C to freeze the aqueous fraction, and then the organic fraction was decanted to another tube and blown to dryness with air at 40°C. Extracted samples were reconstituted with 500 μL of PBS-gel and allowed to stand 30 min. Extraction of tritiated estradiol (estradiol-17β-[2,4,6,7,16,17-3H(N)], New England Nuclear) gave 95% extraction efficiency.

Standards of 120, 60, 30, 15, 8, 4, 2 and 1 pg estradiol/tube were prepared along with tubes to determine the total amount of tritiated estradiol present, buffer control binding (that amount of tritiated estradiol which binds to antibody in the absence of unlabeled estradiol), and nonspecific binding (apparent binding in the absence of antibody). All tubes, including samples, were run in duplicate. After purification on Sephadex LH-20 (Sigma Chemical), tritiated estradiol was diluted to 3000 cpm/50 μL and added to all tubes. Antibody was anti-17β-estradiol-6-BSA from rabbit (Miles-Yeda), diluted to produce 40% buffer control binding and added to all but total and nonspecific tubes. Tubes

were vortex mixed and then incubated overnight at 4°C. Bound tritiated estradiol was separated from the unbound fraction using dextran-coated charcoal solution (0.02% 70,000 MW dextran, 0.25% acid washed and neutralized charcoal, Sigma Chemical Co., in PBS without gelatin). After addition of 1 mL of charcoal solution, all tubes were incubated in ice for 15 min and then centrifuged (2000 × *g* for 10 min). The supernatant was decanted into scintillation vials, scintillation cocktail was added, and each sample counted for 5 min (Beckman LS-2000). Data were analyzed by a computer program using standard logit transformation procedures (Travis 1977).

The assay was validated by three methods. Quantitative recovery of unlabeled estradiol from male plasma gave a slope of 1.015 and $r^2 = 0.99$. Second, dilutions of female plasma were assayed yielding a slope equal to the standard curve (−1.03 versus −0.98). Finally, antibody cross-reactivity with estrone and estriol was determined at 2.4 and <1%, respectively. For a complete description of the technique development and validation, see Mount (1987).

### Phosphoprotein-phosphate Determination

Plasma vitellogenin was estimated in duplicate plasma samples by a phosphoprotein-phosphate assay modified from Wallace and Jared (1968) and van Bohemen et al. (1981). Five millilitres of cold 10% trichloroacetic acid (TCA) was added to 20–40 μL of undiluted plasma in a 12 × 75 mm glass test tube and incubated overnight at 4°C to precipitate plasma proteins. The following day, tubes were centrifuged (2000 × *g* for 10 min) and the supernatant discarded. To remove lipids, the pellet was then washed twice with 2 mL of acetone followed by centrifugation. After the second wash, the pellet was blown to dryness under air. Next the dried pellet was dissolved in 1 mL of 2 N NaOH and incubated in water (about 90°C) for 15 min, liberating the protein-bound phosphate. Tubes were then cooled in an ice bath and neutralized with 167 μL of concentrated HCl. Four millilitres of cold 10% TCA was added and incubated overnight at 4°C to precipitate any remaining proteins. The following day, the tubes were centrifuged and the supernatant decanted into 13 × 150 mm tubes. Duplicate standards of 0, 1, 2, 3, 4, 5, and 6 mg $PO_4$/L in 5 mL of TCA were made, and then, 5 mL of 3% (by volume) HCl was added to both standards and samples. Phosphate in samples and standards was then measured colorimetrically by reaction with ammonium molybdate (S. Boese, Department of Geology, University of Wyoming, Laramie, WY).

Phosvitin (from chicken, Sigma Chemical), a subunit of vitellogenin, was used as a phosphoprotein standard for assay validation. This protein, listed by the manufacturer as approximately 10% phosphate by weight, contained 9.5% phosphoprotein-phosphate as measured by our assay. Validation included incremental recovery of phosphoprotein-phosphate from dilutions of phosvitin ($r^2 = 0.99$) and from male brook trout plasma spiked with phosvitin ($r^2 = 0.99$). In addition, we demonstrated incremental recovery of phosphoprotein-phosphate from dilutions of female brook trout plasma ($r^2 = 0.99$). For a complete description of the technique development and validation, see Mount (1987).

### Statistics

With the exception of survival, only data for female fish are reported. Data were analyzed using one-way analysis of variance (ANOVA). The neutral, high-Ca (1) treatment was considered as the "control" condition for these experiments, and the remaining treatments (2–6) were compared with this group using Dunnett's multiple range test (Dunnett 1964). If data for a particular measure were not normally distributed (chi-square goodness of fit test at $p = 0.01$; Sokal and Rohlf 1969) or had heterogeneous variances (Bartlett–Box $F$ statistic at $p = 0.01$; Neter et al. 1985), $\log_{10}$ or square-root transformations were tried. If the data still failed to meet criteria for normality and homogeneity of variances, the nonparametric Kruskal–Wallis one-way ANOVA was used (Nie et al. 1975). Pairwise comparisons were then made by the method of Zar (1984) for nonparametric comparisons of a control with multiple treatments. Significance of pairwise comparisons for both parametric and nonparametric methods was determined at $p = 0.05$.

TABLE 2. Survival (%) and weight (g) for brook trout over 193-d exposure period (mean ± SEM; sample size = 16 fish/treatment).

| | Treatment | | | | | |
|---|---|---|---|---|---|---|
| | (1) Neutral, high Ca | (2) Neutral, low Ca | (3) Acidic, high Ca | (4) Acidic, high Ca + Al | (5) Acidic, low Ca | (6) Acidic, low Ca + Al |
| Day 41 | | | | | | |
| Survival | 100 | 100 | 100 | 100 | 62.5 | 62.5 |
| Weight | 285 ± 12 | 255 ± 13 | 274 ± 17 | 247 ± 12 | 154 ± 11[a] | 154 ± 10[a] |
| Day 97 | | | | | | |
| Survival | 100 | 100[b] | 100 | 93.8 | 62.5 | 62.5 |
| Weight | 335 ± 17 | 300 ± 18 | 343 ± 20 | 330 ± 20 | 224 ± 20[a] | 150 ± 9[a] |
| Day 147 | | | | | | |
| Survival | 100 | 100 | 100 | 93.8 | 62.5 | 62.5 |
| Weight | 404 ± 21 | 400 ± 24 | 401 ± 22 | 350 ± 26 | 362 ± 40 | 153 ± 14[a] |
| Day 193 | | | | | | |
| Survival | 93.8 | 100 | 100 | 93.8 | 62.5 | 56.2 |
| Weight[c] | 401 ± 23 | 389 ± 22 | 389 ± 22 | 341 ± 23 | 352 ± 43 | 147 ± 15[a] |

[a]Different from control (treatment 1) on same sampling day ($p = 0.05$).
[b]Sample size reduced from 16 to 10 fish by equipment failure.
[c]Weight for female fish recorded before eggs were stripped.

TABLE 3. Plasma Na, osmolality, and hematocrit values for female brook trout on days 41, 97, 147,and 193 (mean ± SEM).

| | Treatment | | | | | |
|---|---|---|---|---|---|---|
| | (1) Neutral, high Ca | (2) Neutral, low Ca | (3) Acidic, high Ca | (4) Acidic, high Ca + Al | (5) Acidic, low Ca | (6) Acidic, low Ca + Al |
| Day 41 | | | | | | |
| Na (meq/L) | 144 ± 5 | 134 ± 9 | 124 ± 9 | 119 ± 11[a] | 121 ± 11[a] | 121 ± 9[a] |
| Osmolality (mosmol/kg) | 331 ± 2 | 334 ± 3 | 322 ± 2[a] | 319 ± 2[a] | 309 ± 4[a] | 307 ± 2[a] |
| Hematocrit (%) | 42 ± 1 | 43 ± 1 | 42 ± 1 | 44 ± 1 | 41 ± 1 | 45 ± 1 |
| *n* | 13 | 9 | 12 | 11 | 9 | 8 |
| Day 97 | | | | | | |
| Na (meq/L) | 147 ± 2 | 150 ± 1 | 145 ± 2 | 146 ± 1 | 145 ± 1 | 128 ± 4[a] |
| Osmolality (mosmol/kg) | 318 ± 4 | 322 ± 2 | 315 ± 1 | 321 ± 3 | 310 ± 3 | 290 ± 3[a] |
| Hematocrit (%) | 41 ± 1 | 41 ± 1 | 39 ± 1 | 45 ± 1 | 36 ± 1[a] | 39 ± 3 |
| *n* | 13 | 9 | 12 | 11 | 9 | 8 |
| Day 147 | | | | | | |
| Na (meq/L) | 145 ± 2 | 145 ± 3 | 140 ± 2 | 136 ± 3 | 139 ± 1 | 136 ± 4 |
| Osmolality (mosmol/kg) | 313 ± 2 | 313 ± 2 | 305 ± 2 | 313 ± 3 | 309 ± 3 | 301 ± 5[a] |
| Hematocrit (%) | 45 ± 2 | 26 ± 2[a] | 25 ± 1[a] | 31 ± 2 | 28 ± 3 | 26 ± 1[a] |
| *n* | 13 | 8 | 12 | 11 | 9 | 8 |
| Day 193 | | | | | | |
| Na (meq/L) | 139 ± 3 | —[b] | 138 ± 3 | 131 ± 4 | 142 ± 1 | 125 ± 2[a] |
| Hematocrit (%) | 29 ± 2 | —[b] | 23 ± 3 | 32 ± 2 | 28 ± 3 | 28 ± 2 |
| *n* | 12 | | 12 | 11 | 9 | 7 |

[a]Different from control (treatment 1) on the same sampling day ($p = 0.05$).
[b]Not measured because of accidental deaths (see text).

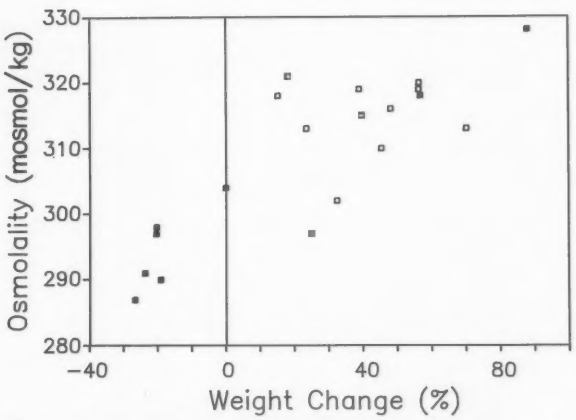


FIG. 1. Relationship between plasma osmolality measured on day 147 and percent change in body weight from day 41 to 193 within the acidic, low Ca + Al treatment (6) (solid squares). Data from control fish (the neutral, high Ca treatment) are shown for reference (open squares).

## Results

### Survival and Growth

Survival was 94–100% in all treatments except the acidic, low Ca and acidic, low Ca + Al treatments (5 and 6) (Table 2); both of these treatments had 37.5% mortality during days 0–16 of exposure. After pH was raised in those treatments on day 16, no further mortalities were observed, except for one fish in the acidic, low Ca + Al treatment (6) that died after spawning.

After 41 days of exposure, mean body weight was lower in both acidic, low Ca treatments (5 and 6) (Table 2). This corresponded with reduced feeding observed in both tanks during this time. Food consumption continued to be low in the acidic, low Ca + Al treatment (6) throughout the exposure; mean body weight in this tank remained essentially unchanged throughout the exposure. In contrast, fish in the acidic, low Ca treatment (5) greatly increased feeding behavior about day 50 and showed steady weight gain throughout the remainder of the experiment. By the end of the experiment, only the acidic, low Ca + Al treatment (6) had a significantly lower mean body weight than fish in the control treatment (1).

### Plasma Na, Osmolality, and Hematocrit

Fish in all acidic treatments (3–6) showed some signs of ionoregulatory impairment at day 41, as evidenced by lower plasma osmolality and Na concentrations (Table 3). Furthermore, fish exposed to low pH and low Ca showed lower plasma osmolality than those exposed to low pH and higher Ca, although this difference is not reflected in plasma Na concentrations. By day 97, however, both plasma osmolality and Na concentrations were essentially equal to control in all but the most severe condition, the acidic, low Ca + Al treatment (6). Once again at day 147, plasma osmolality was lower than control in this treatment; plasma Na concentrations in other acidic treatments tended to be slightly lower than control, although not to the degree that they were on day 41. At the end of the experiment, only the acidic, low Ca + Al treatment (6) had plasma Na significantly lower than control. Equipment failure prevented measurement of plasma osmolality on day 193.

Among fish in the acidic, low Ca + Al treatment (6), reductions in growth appear to be correlated with osmoregulatory status (Fig. 1). Fish able to maintain normal plasma osmolality were the only individuals that increased in body weight during the exposure.

TABLE 4. Plasma estradiol, phosphoprotein-phosphate, and Ca concentrations for female brook trout on days 41, 97, 147, and 193 (mean ± SEM).

| | Treatment | | | | | |
|---|---|---|---|---|---|---|
| | (1) Neutral, high Ca | (2) Neutral, low Ca | (3) Acidic, high Ca | (4) Acidic, high Ca + Al | (5) Acidic, low Ca | (6) Acidic, low Ca + Al |
| Day 41 | | | | | | |
| Estradiol (ng/mL) | 5.1 ± 0.8 | 6.2 ± 0.7 | 6.6 ± 0.9 | 4.3 ± 0.6 | 1.9 ± 0.3[a] | 3.3 ± 0.6 |
| Phosphoprotein-$PO_4$ (mg/mL) | 42 ± 4 | 35 ± 8 | 50 ± 8 | 31 ± 7 | 13 ± 5[a] | 44 ± 9 |
| Ca (meq/L) | 6.8 ± 0.4 | 6.0 ± 0.5 | 7.2 ± 0.5 | 5.8 ± 0.4 | 5.1 ± 0.3[a] | 5.5 ± 0.3 |
| *n* | 12 | 9 | 12 | 10 | 9 | 8 |
| Day 97 | | | | | | |
| Estradiol (ng/mL) | 17.0 ± 2.8 | 12.7 ± 1.6 | 26.9 ± 3.8 | 19.3 ± 2.5 | 12.9 ± 3.8 | 14.2 ± 2.2 |
| Phosphoprotein-$PO_4$ (mg/mL) | 78 ± 5 | 61 ± 6 | 83 ± 9 | 72 ± 7 | 62 ± 6 | 57 ± 9 |
| Ca (meq/L) | 8.4 ± 0.3 | 7.8 ± 0.3 | 11.2 ± 1.0 | 11.6 ± 1.6 | 8.2 ± 0.7 | 10.4 ± 1.3 |
| *n* | 13 | 9 | 12 | 10 | 9 | 8 |
| Day 147 | | | | | | |
| Estradiol (ng/mL) | 73.8 ± 6.6 | 77.1 ± 9.6 | 65.5 ± 7.4 | 63.4 ± 9.6 | 70.0 ± 12.0 | 36.2 ± 8.4[a] |
| Phosphoprotein-$PO_4$ (mg/mL) | 431 ± 66 | 378 ± 44 | 305 ± 33 | 308 ± 62 | 431 ± 97 | 202 ± 36[a] |
| Ca (meq/L) | 12.9 ± 1.2 | 10.0 ± 1.0 | 10.2 ± 0.9 | 8.8 ± 1.1[a] | 11.3 ± 2.2 | 6.6 ± 0.7[a] |
| *n* | 13 | 9 | 12 | 11 | 9 | 8 |
| Day 193 | | | | | | |
| Estradiol (ng/mL) | 0.6 ± 0.1 | —[b] | 0.6 ± 0.1 | 2.0 ± 1.3 | 0.7 ± 0.2 | 0.5 ± 0.1 |
| Phosphoprotein-$PO_4$ (mg/mL) | 193 ± 36 | —[b] | 107 ± 20 | 197 ± 45 | 183 ± 57 | 31 ± 17[a] |
| Ca (meq/L) | 12.4 ± 1.5 | —[b] | 8.8 ± 0.8 | 10.4 ± 1.6 | 11.8 ± 2.23 | 5.3 ± 0.7[a] |
| *n* | 12 | | 12 | 11 | 9 | 7 |

[a]Different from control (treatment 1) on the same sampling day ($p = 0.05$).
[b]Not measured because of accidental deaths (see text).

Hematocrit did not show clear trends across treatments at any of the four sampling days (Table 3), despite the obvious ionoregulatory disturbance experienced in some treatments, particularly the acidic, low Ca + Al treatment (6). There was, however, a decrease in mean hematocrit within all treatments over the course of the experiment. This drop was especially evident between days 97 and 147.

### Plasma Estradiol, Vitellogenin, and Ca

During the course of the experiment, fish in all treatments showed changes in plasma estradiol titers characteristic of vitellogenesis and spawning, rising steadily to the observed peak at day 147 and falling to 2 ng/mL or below after spawning (Table 4). Across treatments, there were relatively small differences in mean concentrations through days 41 and 97, although treatments with low pH and low Ca (5 and 6) tended to have slightly lower concentrations than did high Ca treatments (3 and 4). By the observed peak of yolking (day 147), however, plasma estradiol concentrations within the acidic, low Ca + Al treatment (6) averaged only 49% of control values (Fig. 2a). All other treatments had concentrations similar to control. After spawning, very little estradiol was present in any fish.

In general, plasma vitellogenin (as phosphoprotein-phosphate) and Ca concentrations followed patterns very similar to estradiol throughout the experiment, although concentrations of both vitellogenin and Ca remained somewhat elevated after spawning (Table 4). Once again, fish in the acidic, low Ca + Al treatment (6) showed a disturbance of normal yolking at day 147 (Fig. 2b and 2c); vitellogenin and Ca concentrations averaged only 47 and 51% of those in the control treatment (1). Although yolking was already complete, vitellogenin concentrations in the acidic, low Ca + Al treatment (6) were only 16% of control values at day 193.

### Fecundity and Ovarian Data

All female fish in all treatments produced mature eggs with the exception of two fish, one in each of the two treatments with added Al. These fish had plasma estradiol titers above those measured in male fish, yet did not show the peak of estradiol production at day 147. Neither fish had ovaries containing any mature eggs and were not included in fecundity analyses. Of the mature fish, those in the acidic, low Ca + Al treatment (6) tended to mature slightly later than control fish, but all fish matured within a 2-wk period.

Fecundity data (Table 5) were highly variable; although all low Ca treatments (2, 5, and 6) had fecundity values (mean eggs per female) lower than control fish (33–81%), this reduction was significant only for the acidic, low Ca + Al treatment (6). Mean number of eggs per 100 g of body weight also was lower in these same treatments, but the reductions were smaller than for eggs per female and were not statistically significant. This suggests that some, although not all, of this effect was due to reduced body weight, particularly in the acidic, low Ca + Al treatment (6). Mean egg diameter was not affected by treatment. After spawning, ovaries from fish exposed to low pH and low Ca tended to have more large yolky eggs still embedded in the ovary than did control fish. GSI was, at this time, significantly higher in the acidic, low Ca + Al treatment (6), reflecting the larger numbers of unovulated eggs.

## Discussion

This study has confirmed the hypothesis that long-term, sublethal exposure to combinations of acid, Al, and low Ca can

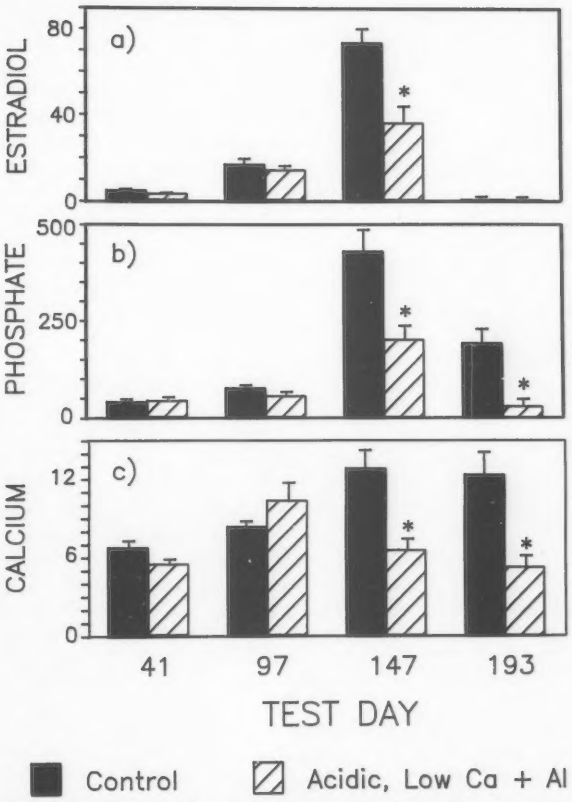


FIG. 2. Comparison of mean (a) plasma estradiol (ng/mL), (b) phosphoprotein-phosphate (μg/mL), and (c) Ca (meq/L) concentrations in female brook trout exposed to the neutral, high Ca treatment (1) and the acidic, low Ca + Al treatment (6) at the four sampling days (error bar = SEM; *different at $p = 0.05$).

alter normal reproductive and osmoregulatory physiology in brook trout. Overall, most of the physiological and toxicological effects observed in this study were found in the acidic, low Ca + Al treatment (6); fish exposed to these conditions displayed a suite of toxicological and physiological abnormalities, including osmoregulatory impairment and marked disturbances of normal vitellogenesis, as well as decreased survival, growth, and feeding. In spite of these problems, these fish did complete oocyte maturation and ovulation. Fecundity was reduced, but this effect was largely due to reduction in growth. The cause of toxicity within the acidic, low Ca + Al treatment (6) appears to have been a combined acid, Al, and low Ca effect, since either raising Ca or lowering Al concentrations largely alleviated the observed responses.

Three other studies have examined the effects of chronic exposure to acidic waters on reproduction in brook trout (Menendez 1976; Tam and Payson 1986; Mount et al. 1988). Menendez found no effect on egg production, while Tam and Payson and Mount et al. did demonstrate reductions in egg production, although these reductions were mediated primarily through reductions in growth. Again in the present study, the only statistically significant reduction in egg production was related to reductions in growth. Data from the present study suggest, however, that low Ca might have an additional effect on egg production, beyond that of growth reductions. Eggs per 100 g of body weight in all low Ca treatments (including pH 6.5) were lower than in any high Ca treatment, 75–83% of that in control fish (Table 5).

Adult brook trout exposed to 0.5 mg Ca/L also have been shown to produce eggs which are more sensitive to incubation in low Ca water than control eggs (Mount et al. 1988), although similar data are not available for the present study. Despite low vitellogenin production in fish exposed to low Ca, low pH, elevated Al conditions, ascribing sensitization of progeny to lowered vitellogenin production would be tenuous. This low Ca sensitization appears to be independent of pH and Al (Mount et al. 1988), while lowered vitellogenin production in the present study was not (Table 4). Making a credible connection would require simultaneous measurement of progeny sensitivity and vitellogenin production, or perhaps egg protein content.

As discussed above, inhibition of oocyte maturation has been suggested as a mechanism for reproduction failure in acidic waters (Ruby et al. 1977). In addition, some field evidence suggests that this may occur in wild brook trout populations (Frenette and Dodson 1984). But as in our previous study (Mount et al. 1988), fish in the present study produced mature oocytes at all treatment combinations. In general, fish exposed to low pH, high Al, and low Ca did have 50–100 mature oocytes still embedded in the ovary after manual spawning, as reflected in the significantly higher GSI of the residual ovaries (Table 5); this same tendency has been observed also in acid-stressed rainbow trout (*Salmo gairdneri*) (Weiner et al. 1986). Nevertheless,

TABLE 5. Fecundity and ovarian measurements on female brook trout at spawning (mean ± SEM).

| | Treatment | | | | | |
|---|---|---|---|---|---|---|
| | (1) Neutral, high Ca | (2) Neutral, low Ca | (3) Acidic, high Ca | (4) Acidic, high Ca + Al | (5) Acidic, low Ca | (6) Acidic, low Ca + Al |
| Eggs per female | 1456 ± 108 | 1175 ± 87 | 1398 ± 277 | 1483 ± 102 | 990 ± 153 | 483 ± 135[a] |
| Eggs per 100 g | 377 ± 29 | 312 ± 23 | 347 ± 52 | 420 ± 21 | 296 ± 27 | 283 ± 60 |
| Egg diameter (mm) | 4.41 ± 0.07 | 4.52 ± 0.08 | 4.37 ± 0.04 | 4.37 ± 0.06 | 4.36 ± 0.05 | 4.38 ± 0.10 |
| Residual ovary[b,c] | 1.3 ± 0.2 | 1.4 ± 0.3 | 1.5 ± 0.2 | 1.2 ± 0.2 | 1.8 ± 0.3 | 2.2 ± 0.4 |
| Ovary weight (g)[c] | 4.0 ± 1.0 | 5.9 ± 2.2 | 4.4 ± 1.2 | 3.2 ± 1.0 | 4.0 ± 0.8 | 4.8 ± 1.6 |
| GSI (%)[c,d] | 1.1 ± 0.3 | 1.5 ± 0.6 | 1.1 ± 0.2 | 0.9 ± 0.3 | 1.1 ± 0.2 | 3.0 ± 1.1[a] |
| *n* | 13 | 9 | 11 | 10 | 8 | 6 |

[a]Different from control (treatment 1) at $p = 0.05$.
[b]Ovaries classified according to the number of unovulated eggs remaining within the ovary (1 = <10; 2 = 10 – 50; 3 = >50).
[c]Measured on ovaries removed after spawning at day 193.
[d]Calculated as [(ovarian weight in grams)/(body weight in grams)] × 100%.

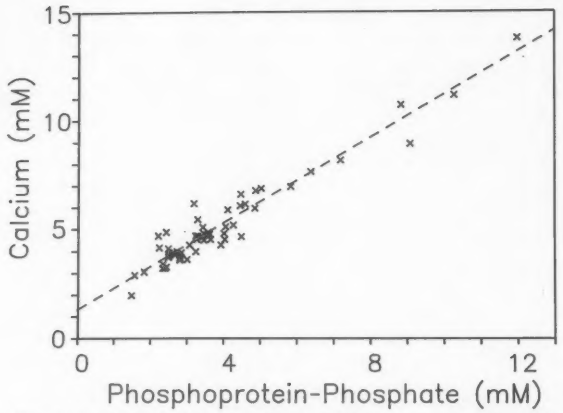


FIG. 3. Relationship between plasma vitellogenin (measured as phosphoprotein-phosphate) and plasma Ca across all treatment groups. Regression equation is (mM Ca) = 0.9814 mM phosphoprotein-phosphate) + 1.34 ($r^2$ = 0.93).

the oocytes remaining within the ovary were of normal diameter and appearance, with no evidence of inhibited oocyte growth and atresia, as described by Ruby et al. (1977). Even if these eggs were never ovulated, it would represent a comparatively small reduction in fecundity.

Two female fish in elevated Al treatments did not mature sexually. While we cannot completely discard the potential importance of such an effect, we feel that this response was too limited to base any conclusions on. These fish represented only 11% of the Al-exposed female fish, which would cause a much smaller reduction in fecundity than that caused by reduced growth. Moreover, test fish were entering their first reproductive season, and sexual maturity in brook trout is often not achieved until >2 yr of age (Saunders and Power 1970; Frenette and Dodson 1984).

Ionoregulatory disturbance in response to acute acid and Al stress has been well documented in the literature (see Wood and McDonald 1987 for review). Mortality is attributed mainly to critical loss of body electrolytes leading to hemoconcentration and circulatory collapse, although respiratory impairment may contribute significantly at certain exposure combinations. In essence, survival through acute challenges relies upon the fish's ability to reduce ion efflux while restoring sufficient uptake to maintain body ion levels. Consequently, the majority of deaths occur during the first days of exposure.

Long-term responses to acid/Al exposure have been studied less intensively. Experiments by various researchers have demonstrated partial or complete compensation for initial plasma ion losses over the course of several weeks. Brook trout exposed to natural water at pH 4.6 had plasma Na concentrations lower than control, even after a year of exposure (Leivestad et al. 1976). In contrast, brook trout exposed to pH 5.2 in the laboratory showed complete recovery of plasma ions after 2 mo of exposure (Wood et al. 1988). Our data suggest that brook trout do compensate for initial ion losses and that the degree of recovery is dependent on the severity of the exposure (Table 3). All fish exposed to low pH showed significantly decreased plasma sodium concentrations after 41 d of exposure. But after 97 d of exposure, only those fish exposed to the harshest conditions (low pH, low Ca, and elevated Al) failed to compensate for initial Na losses. Regardless of these ionic disturbances, at no time did stressed fish appear near hemoconcentration and circulatory failure (Milligan and Wood 1982; Giles et al. 1984), as hematocrit stayed near or below control values at all times. In fact, hematocrit values declined steadily throughout the exposure, which has been reported previously for female brook trout during vitellogenesis (McCormick and Naiman 1984).

Throughout vitellogenesis, control fish underwent changes in plasma estradiol and vitellogenin concentrations typical of brook trout (Tam et al. 1986b). Absolute concentrations of both were somewhat higher in our study (73.8 ng estradiol/mL and 431 μg phosphoprotein-phosphate/mL) than those reported by Tam et al. (40 ng/mL and 294 μg/mL, respectively), but this could easily be due to strain differences, sampling times, or analytical techniques. In the present study, however, comparing phosphoprotein-phosphate and Ca concentrations yields a slope essentially equal to 1 (Fig. 3), indicating that Ca and phosphate groups are present in a 1:1 ratio, a feature previously demonstrated for amphibian vitellogenin (Wallace 1970). This, combined with the close correlation between the parameters ($r^2$ = 0.93), suggests that our vitellogenin values are indeed correct.

In contrast with the ionoregulatory parameters, differences in the reproductive physiology between control fish and fish exposed to low pH, low Ca, and elevated Al did not emerge until day 147 of exposure (Table 4). The effects on plasma estradiol, vitellogenin, and Ca concentrations were, however, much more dramatic than those for plasma Na and osmolality. On average, fish exposed to low pH, low Ca, and elevated Al conditions appear to have been synthesizing only half as much yolk protein as control fish, assuming no differences in deposition rates at the ovary. Nonetheless, we found little expression of this phenomenon in other reproductive measures. These same fish also produced 75% as many eggs per 100 g of body weight as control fish, but this does not appear to be a consequence of the lower vitellogenin concentrations; the acidic, low Ca treatment (5) had similarly reduced egg production per body weight, but had vitellogenin concentrations equal to those in control fish. Changes in the degree of phosphorylation of the vitellogenin in stressed fish would reduce apparent vitellogenin concentration, since we quantified vitellogenin on the basis of phosphoprotein-phosphate. This also seems unlikely, since plasma estradiol and Ca concentrations were reduced by essentially the same percentage as was vitellogenin. Temporal differences in the maturation of control and stressed fish might also explain the observed differences in reproductive parameters. Based on the rate of concentration change in control fish (Fig. 2), however, a 2-wk offset alone would not seem sufficient to explain a 50% reduction in yolk production.

The most comparable work to the present study is that of Tam et al. (1986a). In their study, brook trout were exposed to acidified hard water (pH 4.48) for 10 mo, with periodic measurements of plasma estradiol and vitellogenin (phosphoprotein-phosphate), as well as cortisol and glucose. These fish developed some symptoms similar to those from the acidic, low Ca + Al treatment in our study (e.g. decreased growth), but did not differ from control fish in plasma estradiol or vitellogenin concentrations. In a separate experiment, however, fish surgically implanted with estradiol capsules and exposed to low pH had lower vitellogenin production than did similarly implanted fish held in neutral water. During the acid exposure, these fish also exhibited elevated plasma glucose, suggesting stress. Based on evidence from other experiments, the authors inferred that elevated plasma cortisol, while not measured, was responsible for the increased plasma glucose. They further suggested that

the effect of low pH on yolk deposition was due to cortisol-mediated changes in hepatic carbohydrate metabolism, which reduced vitellogenin production. However, the reported effects of stress on reproduction in fish are quite varied and inconclusive (Greenberg and Wingfield 1987). In fact, glucocorticoids have been reported to actually *potentiate* the stimulatory effect of estradiol on vitellogenin production in the catfish (*Heteropneustes fossilis*) (Lamba et al. 1983); many species have naturally elevated glucocorticoids during reproduction, even in the absence of other perturbations (Greenberg and Wingfield 1987).

In our study, clear effects of acid/Al stress on vitellogenesis did not develop until the peak of vitellogenesis. As Tam et al. (1986a) pointed out, acid-exposed fish in their experiment showed signs of acclimation to the acid stress by this time; hence, the lack of effect on vitellogenesis. But our data do not support the conclusion that depression of vitellogenesis is due to effects on liver metabolism. In affected fish, mean plasma estradiol concentrations were reduced by almost exactly the same percentage as were vitellogenin concentrations. Consequently, we suggest that in chronically exposed, intact fish, the interruption in normal reproductive physiology lies earlier in the vitellogenic process, possibly in gonadotropin or estradiol synthesis and secretion. Since gonadotropin is also responsible for initiating ovulation, the observed retention of eggs within the ovary further supports this hypothesis. Such a response might be caused directly by ionoregulatory insufficiency, or mediated through cortisol or other physiological responses to stress (Greenberg and Wingfield 1987). In addition, one cannot discount the effects of starvation upon the anabolic capacity of these stressed fish. Nutritional state has been shown to affect vitellogenesis in mummichog (*Fundulus heteroclitus*) (Wallace and Selman 1980).

In summary, brook trout exposed to low pH, low Ca, and elevated Al exhibited a toxic syndrome consisting of several symptoms: ionoregulatory impairment (decreased plasma sodium and osmolality), decreased feeding behavior, decreased growth, abnormal vitellogenesis, and decreased egg production. Although the cause and effect relationships involved are not clear, we believe ionoregulatory stress to be a likely cause for the other upsets, particularly since it develops so rapidly upon initiation of exposure (<1 h; Booth et al. 1988). Fish that were able to maintain higher plasma osmolality in the face of acid/Al/Ca stress gained weight (Fig. 1) and showed much less severe reductions in other parameters. In light of these data, we suggest that physiological disturbances, such as those shown for fish exposed to low pH, low Ca, and elevated Al, would likely take place in fish exposed to any combination of acid, Al, and Ca severe enough to cause ionoregulatory insufficiency.

While disturbances of reproductive physiology are clearly possible in brook trout exposed to acid, Al, and low Ca, they were associated with weight losses that alone would lead to loss of affected individuals, even without reproductive impairment. We cannot discern from the present study whether it is possible for brook trout to be stressed sufficiently to interrupt vitellogenesis without affecting survival of the adults directly. Evidence from studies of white suckers, flagfish, rainbow trout, and fathead minnows (*Pimephales promelas*) do indicate that reproduction in those species can be affected at water chemistries that allow for survival and growth of adult fish (Beamish et al. 1975; Hutchinson and Sprague 1986; Weiner et al. 1986; Mount 1973). But for brook trout, the results of this study, combined with those of the preceding study (Mount et al. 1988), support the assertion that the most important effects of surface water acidification on adult brook trout are on survival and growth, rather than egg development.

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# Morphometric Changes in Gill Secondary Lamellae of Brook Trout (*Salvelinus fontinalis*) After Long-Term Exposure to Acid and Aluminum

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Adult brook trout (*Salvelinus fontinalis*) were exposed for 147 d to three different combinations of acid and Al in soft water (2 mg Ca/L). Samples of gill tissue from each of the three exposure conditions (pH 6.64 + 0 μg Al/L, pH 4.91 + 4.3 μg Al/L, and pH 4.45 + 393 μg Al/L) were examined by light microscopy using high-resolution techniques and morphometric analysis. As compared with fish in control conditions (pH 6.64 + 0 μg Al/L), exposure to pH 4.91 without Al significantly increased volume density of lamellar chloride cells. Low pH and elevated Al (pH 4.45 + 393 μg Al/L) resulted in increased diffusion distance, white blood cell infiltration of the lymphatic space of the gill tissue (an indicator of tissue damage), and a dramatic increase in lamellar dense cells. These results are consistent with the physiological data that show ionoregulatory stress and decreased respiratory efficiency as typical responses of brook trout exposed to low pH and combinations of low pH and elevated Al.

Pendant 147 d on a exposé des adultes d'omble de fontaine (*Salvelinus fontinalis*) à trois différentes combinaisons d'acide et d'Al dissous en eau douce (2 mg/L de Ca). On a examiné des échantillons d'ouïe prélevés chez des individus exposés aux trois différentes conditions environnementales (pH 6,64 + 0 μg/L d'Al, pH 4,91 + 4,3 μg/L d'Al et pH 4,45 + 393 μg/L d'Al) au microscope optique à résolution élevée puis on a effectué une analyse morphométrique. Par rapport aux poissons témoins (pH 6,64 + 0 μg/L d'Al), l'exposition à un pH 4,91 en absence d'Al a amené une nette augmentation de la densité volumétrique des cellules à chlorure lamellaires. L'exposition à un faible pH et à une teneur élevée en Al (pH 4,45 + 393 μg/L d'Al) a entraîné une augmentation de la distance de diffusion, de l'infiltration des cellules sanguines blanches dans l'espace lymphatique des ouïes (un indicateur de lésions tissulaires) ainsi qu'une augmentation marquée du nombre de cellules denses lamellaires. Les résultats obtenus s'accordent avec les données physiologiques qui révèlent que le stress ionorégulatoire et une efficacité respiratoire abaissée sont des réactions typiques de l'omble de fontaine exposé à un faible pH et à des combinaisons de faibles pH et de teneurs élevées en Al.

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The mechanisms by which low pH water and combinations of low pH water and elevated Al concentrations are toxic to fish are not completely understood. Understanding these mechanisms, however, would improve our ability to predict the fate of fish populations exposed to acidified surface waters. pH levels and Al concentrations vary with location, along with other variables such as Ca concentration, fish species, and species strain. However, laboratory toxicity studies can only address a limited number of variables, making it difficult to predict toxic effects in feral fish populations using laboratory toxicity results. Physiological and histological information is needed to understand the effects of these variables at the level of the organism. Ultimately, this information may enable predictions about the health of fish populations in the field (Rankin et al. 1982).

The main physiological problems observed in fish exposed to low pH and elevated Al are ionoregulatory disfunction, reduced oxygen uptake, and a drop in blood pH (acidosis) (Fromm 1980; Spry et al. 1981; Leivestad 1982; Booth et al. 1988; Wood et al. 1988). Given the vital ionoregulatory (Evans 1980), respiratory (Hughes 1972; Hughes and Morgan 1973), and acid–base regulation (Haswell et al. 1980) functions of the gill, it is logically the tissue to examine for pathological changes that may result in such physiological problems. Daye and Garside (1976) have documented gill damage in brook trout (*Salvelinus fontinalis*) exposed to low pH water for about 7 d. In another laboratory study, Leino and McCormick (1984) demonstrated an increase in chloride cell numbers on the secondary lamellae of fathead minnows (*Pimephales promelas*) exposed to acidic conditions for 129 d. With exposure to low pH, they also demonstrated an increase in apical pit formation in the chloride cells. Their quantitative study suggests that the chloride cell function may be altered during acid exposure. Field studies of brook trout from three acidified lakes have documented an increase in the number of chloride cells and general

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epithelial damage in the gills (Chevalier et al. 1985). The increase in chloride cell numbers is consistent with the laboratory results, but the epithelial damage is difficult to interpret because generalized epithelial damage of the gill is a common pathological response to several toxicants (Mallatt 1985).

The aim of this study is to quantitatively examine changes in the morphology of the secondary lamellae of brook trout gills as they relate to the physiological problems observed during acid stress. This approach will determine if the cellular composition or the dimensional characteristics of the secondary lamellae change in response to exposure to low pH and elevated Al.

## Materials and Methods

### Exposure

Adult brook trout, obtained from the Twin Buttes Trout Hatchery, Durango, Colorado, were transported to Laramie, Wyoming, and acclimated over a 7-d period to pH 6.5 water with 2 mg Ca/L. They were held in these conditions for 4 wk prior to the beginning of the experiment.

Well water was softened using NaCL prior to treatment by reverse osmosis. Reverse osmosis product water was then deionized using separate cation- and anion-exchange columns (Continental Water Systems, Denver, Colorado). The pH of the deionized water was continually monitored and adjusted to 6.5 (Leeds and Northrup pH analyzer/controller model 7083) using either $H_2SO_4$ or NaOH, as necessary. Na (1 mg/L) and Ca (0.5 mg/L) were added as chloride salts by a metering pump (Fluid Metering, Inc.). The pH 6.5 water was split into head tanks for final pH adjustment. The pH was lowered for the exposure groups using $H_2SO_4$. The pH-adjusted water from each head tank was delivered to continuous-flow serial dilutors where Al and Ca were added as chloride salts by Mariotte bottles. Temperature was varied from 9 to 14°C and photoperiod was adjusted biweekly to simulate natural annual cycles.

The fish were randomly distributed into 340-L round, insulated fiberglass tanks (Frigid Units, Inc.) resulting in 14 fish per tank. The experiment began with the adjustment of Ca to the nominal concentration of 2 mg/L followed by lowering the pH to 4.8 (nominal) over several days. Once pH adjustment was completed, Al was added to reach the nominal concentration of 486 μg/L. The addition of Al is considered the beginning of the exposure. The exposure continued for 147 d.

The three conditions examined in this study are part of a larger toxicity study (see exposure 1 in Mount et al. 1988) which employed 27 combinations of pH, Ca, and Al. These three conditions, using nominal exposure values (1) pH 6.5 + 0 μg Al/L, (2) pH 4.8 + 0 μg Al/L, and (3) pH 4.8 + 486 μg Al/L will be referred to as (1) control, (2) low pH, and (3) low pH + Al, respectively.

Exposure water from each tank was analyzed for pH, Ca, Al, and Na. pH was measured weekly (Sargent-Welch pH Meter model LS equipped with an Orion Ross combination electrode) for all tanks. Ca and Na were only measured once during the exposure. Al was measured three times and is reported as total Al. All three cations were measured by atomic absorption spectroscopy using flame or graphite furnace, as necessary (Perkin-Elmer model 2380).

### Tissue Preparation

Five fish from each of the three exposure groups were sampled at the end of the experiment. Fish were killed by a blow behind the head. A sample of the second gill arch from the right side was immediately excised, fixed, and processed for epoxy embedment. Thin sections (1 μm) were cut (Sorvall Instruments model Porter-Blum MT-2B), stained with 0.1% Azure B, and examined and photographed with a light microscope (Carl Zeiss Photomicroscope I) (Johnson and Bergman 1988).

TABLE 1. Categories of lamellar structures used for point counting and volume density estimates.

| Category | Characteristics |
|---|---|
| Pillar/capillary | Pillar cell bodies and flanges, blood lacunae, and marginal channels |
| Epithelium | Pavement epithelium and nondifferentiated cells, not including chloride cells |
| Empty lymphatic space | Empty space between pavement cells and nondifferentiated cells |
| White blood cell | All white blood cells found within the lymphatic space |
| Lamellar chloride cell | Chloride cells only |
| Lamellar dense cell | Dense cells only |
| Total lymphatic space[a] | Sum of empty lymphatic space and white blood cell categories |
| Total lamellar chloride cell[a] | Sum of lamellar chloride cell and lamellar dense cell categories |

[a]Category consisting of a sum of previously defined categories.

### Morphometry and Stereology

All of the lamellae on one side of one filament were included in a series of high-magnification photomicrographs. The micrographs were printed at 1072× and a montage was assembled. Overlapping areas of each micrograph were noted. The nonoverlapping images of the 8 × 10 in. micrographs were analyzed.

Lamellar thickness and diffusion distance can be directly measured. However, since the outermost margins of the lamellae are not uniform, a random sampling procedure was used to avoid biasing the sample toward either minimum or maximum values. A sampling grid was used, similar to that used by Weibel (1963), consisting of randomly placed lines, each 25 mm long, plotted on transparent film. The test grid was placed over each micrograph and wherever a line of the grid intersected the lamellae, measurements were made using a digitizing tablet (Houston Instruments) interfaced to an IBM-XT computer equipped with a morphometric software package (Bioquant, R and M Biometrics, Inc.). Lamellar thickness was simply the perpendicular distance across the lamella. Diffusion distance was measured as the distance along a perpendicular line from the point of intersection to the blood lacuna. If the line intersected a pillar cell rather than the blood space, the measurement was made from the external medium to the nearest blood lacuna.

The secondary lamellae of brook trout are composed principally of four cell types, as described for other fish gills (Morgan and Tovell 1973; Laurent 1982; Laurent and Dunel 1980; Newstead 1967): (1) pillar cells, with flanges that define the interconnecting capillary network of the gill, (2) nondifferentiated cells (sometimes referred to as the inner epithelial layer)

which surround the pillar cell/capillary network and are adjacent to the basement membrane, (3) pavement cells which envelop the entire lamellar structure to form the majority of the mucosal surface, and (4) chloride cells which normally reside in the interlamellar epithelium of the filament. Mucous cells were never noted in the lamellar epithelium. Because of its prevalence, a cell similar to the chloride cell, the dense cell (Morrison 1979), was considered a separate cell type in this study. Other compartments of the secondary lamellae of interest include the capillary space or blood lacuna and the lymphatic space. The lymphatic space is between the pavement epithelium and the nondifferentiated cells. The lymphatic space often contains white blood cells.

A stereological technique was used to quantify the composition of the secondary lamellae by determining the volume density of the different cell types and compartments (Weibel 1979). Volume density is a percentage which is determined by imposing a sampling grid over the micrograph, counting the grid points for each cell type or compartment of interest, and dividing by the total number of grid points. However, standard stereological grids of uniform spacing are not appropriate for anisotropic tissue such as the gill. Therefore, a random grid was generated and plotted on a transparent sheet. This grid was placed over each micrograph to determine the volume densities of six lamellar constituents defined in Table 1. In addition, two more lamellar constituents were calculated from the original six categories (Table 1).

### Statistics

The mean diffusion distance, harmonic mean of the diffusion distance, and the mean lamellar thickness were calculated for all five fish in each of the three exposure groups. The number of measurements for each fish varied depending on the size of the gill. Means for diffusion distance and harmonic mean of diffusion distance were based on 64–256 measurements. Means for lamellar thickness were based on 32–128 measurements. For each exposure group, a mean ($n = 5$) was calculated from the individual means. Volume densities were also calculated for all five fish in each of the three exposure groups. For each exposure group, a mean ($n = 5$) was calculated from the individual volume density for each category defined in Table 1.

Summary statistics were generated and the $F_{max}$ test was applied to test for heterogeneity of variances. If heterogeneous, the data was transformed for use in analysis of variance. A square root transformation was used on lamellar thickness data. All volume density data were transformed by taking the arcsine of the square root of each ratio (Sokal and Rohlf 1981). In the case of lamellar chloride cell volume density, the arcsine transformation was squared. All data were then analyzed by analysis of variance ($p < 0.05$). If significant, pairwise comparisons were made using the Scheffe procedure ($p < 0.05$).

## Results

### Toxicology

Survival between the control, low pH, and low pH + Al exposure groups did not differ significantly (Table 2). However, growth (change in mean weight from day 0 to 63) was significantly affected. Fish from the control and low pH conditions had similar increases in weight, 65 and 64%, respectively. In contrast, fish from the low pH + Al condition lost an average of 2% body weight (Table 2).

### Chemistry

Since Na was added to the system before the exposure water was split into head tanks for pH adjustment, measurements of Na were averaged across all tanks. Na was measured at 11.0 mg/L (± 0.1 SEM; $n = 25$), several times higher than the 1 mg/L nominal value. pH, Ca, and Al measurements approximated the nominal values (Table 2).

### Histopathology

Dimensional characteristics of the secondary lamellae changed significantly in the low pH + Al exposure group (Fig. 1). The mean diffusion distance in the low pH + Al group was largest at 7.52 μm, representing a significant increase of 88% over the control group (4.03 μm) and 67% over the low pH group (4.48 μm). Calculations of the harmonic mean of the diffusion distance yielded similar results to the arithmetic mean (Fig. 1). Mean lamellar thickness was not significantly different among the three test conditions.

The composition of the secondary lamellae changed significantly in all but two of the categories defined in Table 1. Volume density of the pillar capillary network decreased in the low pH + Al group relative to the control and low pH groups (Table 3). Epithelial volume densities were not significantly different among groups (Table 3).

In the lymphatic group, which includes empty lymphatic space, white blood cells, and total lymphatic space classifications, only the white blood cell volume density changed significantly among treatment groups. This was significantly increased in the low pH + Al when compared with the control (Fig. 2).

In the chloride cell group, which includes lamellar chloride cells, lamellar dense cells, and total lamellar chloride cells, significant differences were found in all three classifications (Fig. 3). Lamellar chloride cell volume density of the low pH group was roughly double that of either the control or low pH + Al group, indicating hyperplasia or hypertrophy of chloride cells in response to low pH but not to low pH + Al. Lamellar dense cell volume density differed significantly among all treat-

TABLE 2. Toxicology and chemistry data from the three exposure groups used in this study ($\bar{x}$ = mean; SEM = standard error of the mean; $n$ = sample size). — = not available.

| Exposure group | Survival (%)[a] | Growth (%)[b] | pH | | | Ca | | | Al | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | $\bar{x}$ | SEM | $n$ | $\bar{x}$ | SEM | $n$ | $\bar{x}$ | SEM | $n$ |
| Control | 86 | 65 | 6.64 | 0.06 | 17 | 2.0 | — | 1 | — | — | — |
| Low pH | 100 | 64 | 4.91 | 0.12 | 17 | 1.6 | — | 1 | 4.3 | 1.9 | 3 |
| Low pH + Al | 79 | −2 | 4.45 | 0.06 | 17 | 1.5 | — | 1 | 393.0 | 5.0 | 3 |

[a]Survival from day 0 to 63.
[b]Calculated as (mean wt. day 63 − mean wt. day 0)/mean wt. day 0 × 100.

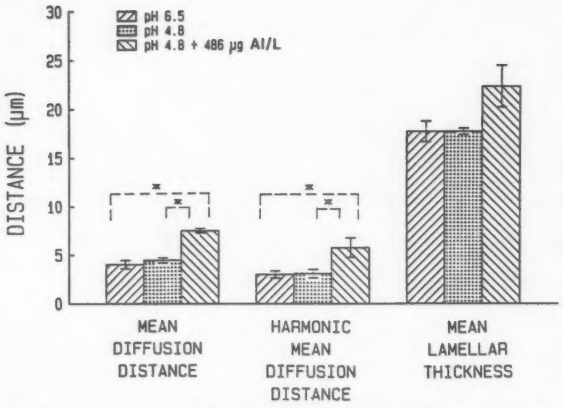


FIG. 1. Arithmetic mean of diffusion distance, harmonic mean of diffusion distance, and mean lamellar thickness for gill secondary lamellae from control, low pH, and low pH + Al exposed brook trout. Error bars represent ±1 SEM ($n$ = 5). Broken lines with asterisks indicate significant differences ($p \leq 0.05$).

ment groups. In the low pH and low pH + Al groups, dense cell volume densities were 2.2 and 10.1%, respectively. Both were significantly higher than that of the control group at 0.4%. Total lamellar chloride cell volume density reflected these changes, differing significantly among all treatment groups. The low pH + Al group was highest at 14.6%, with the low pH and control groups at 11.2 and 4.3%, respectively.

The changes noted in the secondary lamellae that resulted in the morphometric and stereologic observations described above were observable qualitatively (Fig. 4). The photomicrograph of the control lamella is representative of the lamellae of control gills. The photomicrographs of the low pH and the low pH + Al lamellae are of areas chosen to demonstrate the types of pathology present. In these two exposure groups, gradations from the control morphology to that in the micrographs were observed.

## Discussion

The importance of the brook trout gill in respiration and ion regulation is well established. The gill is equipped with several structural features to accomplish these essential physiological functions. These structural adaptations include surface area amplification and large surface to volume ratio, minimal blood/water barrier to facilitate gas exchange, highly vascularized tissue, and the presence of cells thought to be important in ionoregulation. Whenever these morphological characteristics are shown to change in response to some altered environmental condition, it can be suspected that the respiratory and ionoregulatory capacities of these fish also change. Some euryhaline fish, for example, readily adapt to freshwater, seawater, and 2× seawater (Karnaky 1980). Since these exposures represent drastic changes in the osmolality of the external environment, osmoregulatory mechanisms which work to maintain homeostasis should change. The changes should be reflected in functional and structural measures. In fact, such exposures have elicited changes in $Na^+$-$K^+$-ATPase activity (functional measure) and chloride cell numbers and morphology (structural measures) in gill, opercular, and pseudobranch tissue of teleosts (Laurent and Dunel 1980; Karnaky 1980; Philpott 1980), underscoring the inherent connection of structure and function.

When dealing with toxic environmental conditions, structural changes in tissue may be classified into two types. One is the result of the direct toxic effect of the pollutant, as in degeneration and necrosis. The second is a result of compensatory mechanisms that deal with the environmental stressor, as in chloride cell hyperplasia in response to an osmotic challenge. In this study, both of these types of structural responses are noted.

Changes in diffusion distance are mainly the consequence of several cytological changes. White blood cell infiltration into the lymphatic space under the pavement epithelium and chloride cell and dense cell proliferation on the lamellae account for the majority of the increases in these measures. Pavement and nondifferentiated cell hypertrophy, although not measured in this study, could also contribute to diffusion distance. However, based on qualitative observations in this study, the contribution of epithelial hypertrophy on diffusion distance would be minimal compared with the effects of white blood cell infiltration and chloride cell proliferation. In any case, the diffusion distance measure as an indicator of the diffusion barrier is important. The substantial increase in the harmonic mean of the diffusion distance in the low pH + Al group demonstrates a potential for respiratory insufficiency, which would naturally affect the general health of the fish. This is especially true if the fish is required to exercise vigorously, creating a high respiratory demand.

Of the measures made in this study, white blood cell infiltration (as measured by white blood cell volume density) is the best indicator of generalized tissue damage. Only the low pH + Al group exhibited symptoms of actual tissue damage; low pH alone did not elicit this response. This is consistent with the toxicological data which demonstrate significant growth effects present only in the low pH + Al group.

The increase of lamellar chloride cells in the low pH groups are important, since exposure to low pH, even in the absence of gross toxicological effects, causes initial ion losses, followed by a return to normal plasma ion levels over a period of several days (Booth et al. 1988; Wood et al. 1988). One would expect compensatory mechanisms dealing with ion regulation to develop in that time period if the fish were to survive. Laurent and Dunel (1980) demonstrated that a proliferation of chloride cells on the secondary lamellae of trout placed in deionized water can take place within 1 wk. In addition, Leino and McCormick (1984) demonstrated chloride cell proliferation in

TABLE 3. Volume densities (mean ± SEM) of the pillar/capillary network and epithelial cells in the secondary lamellae of brook trout after 147 d of exposure to three conditions ($n$ = 5).

| Category | Control (%) | Low pH (%) | Low pH + Al (%) |
|---|---|---|---|
| Pillar/capillary network | 59.6 ± 1.9 | 53.1 ± 3.4 | 38.0 ± 4.2[a] |
| Epithelial cells | 25.9 ± 1.6 | 24.8 ± 1.7 | 24.8 ± 1.3 |

[a]Significantly different ($p \leq 0.05$).

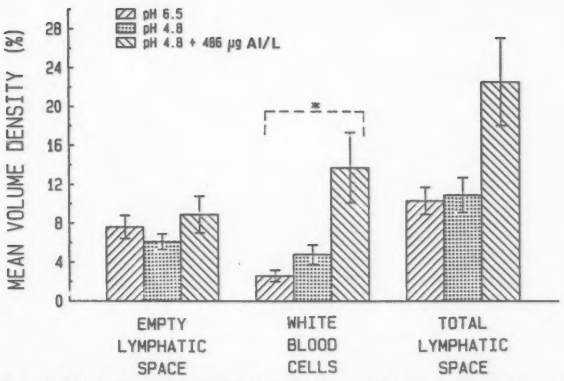


FIG. 2. Mean volume densities for empty lymphatic space, white blood cells, and total lymphatic space for gill secondary lamellae from control, low pH, and low pH + Al exposed brook trout. Error bars represent ± 1 SEM ($n = 5$). Broken lines with asterisks indicate significant differences ($p \leq 0.05$).

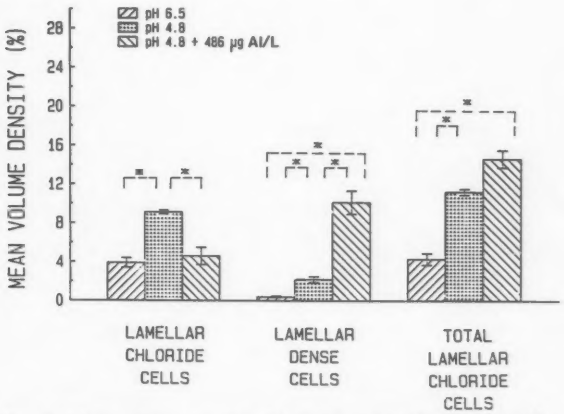


FIG. 3. Mean volume densities for lamellar chloride cells, lamellar dense cells, and total lamellar chloride cells for gill secondary lamellae from control, low pH, and low pH + Al exposed brook trout. Error bars represent ± 1 SEM ($n = 5$). Broken lines with asterisks indicate significant differences ($p \leq 0.05$).

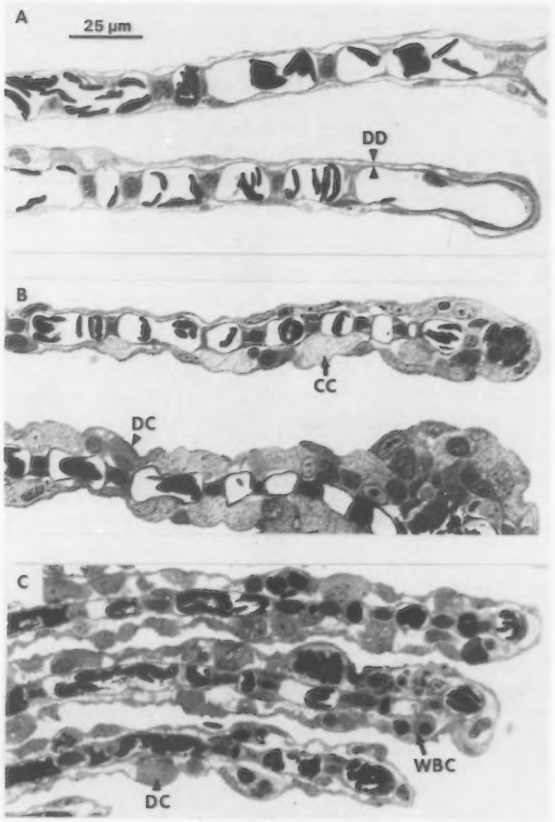


FIG. 4. Photomicrographs of the secondary lamellae illustrating changes in each exposure group. (A) Control; note minimal diffusion distance (DD), lack of chloride cells, and lack of white blood cells; (B) low pH; note predominance of chloride cells (CC) and occasional dense cells (DC); (C) low pH + Al; note increased diffusion distance, extensive proliferation of dense cells (DC), and white blood cell (WBC) infiltration of the lymphatic space.

fathead minnows exposed to pH 5.0 water. While no direct evidence on the specific function of the chloride cells in freshwater-adapted brook trout is available, these observations suggest that chloride cells perform some ion conserving or sequestering function and proliferate in the face of ionic challenge, as in exposure to low pH. The development of this apparent morphological acclimation should parallel the recovery of ion balance with time, if the two phenomena are linked. This study cannot address this question, as the exposure time prior to sampling was far longer than the time required to recover ion balance.

The lamellar dense cell was delineated from the chloride cell in this study. Both cell types have features in common, including apical exposures restricted by overlying layers of pavement epithelium, numerous mitochondria, dense apical regions just below the mucosal membrane, and basally located nuclei. However, using Azure B for light microscopy and methanolic uranyl acetate for electron microscopy, dense cells stain darker. Dense cells contain several 0.5-µm vesicles which are light and electron translucent; vesicles like these are seldom observed in typical chloride cells. Dense cell nuclei are more punctate in appearance, while chloride cell nuclei are smooth. Finally, lamellar dense cells do not contact the basement membrane, whereas most chloride cells do.

Whether the lamellar dense cell represents a different cell type, a functional variant of the chloride cell, or a degenerate chloride cell is unclear. As a different cell type or a functional variant of the chloride cell, it could be the result of an Al-induced change in cell type or function, since dense cell proliferation occurred mostly with Al exposure. This phenomenon would be interesting as a possible compensatory mechanism related to the toxicity of Al at low pH. As a degenerate chloride cell, this cell could be the direct result of the cytotoxic effects of Al. In either case, it is clear that these cells predominate in the low pH + Al group and represent an extensive and fundamental alteration of lamellar morphology. The nature of these cells and the contents of the abundant vesicles are problems that should be pursued.

In summary, exposure of adult brook trout to low pH and a combination of low pH and elevated Al caused morphological changes that are consistent with toxicological and physiological

observations. In the low pH group, no decreases in growth or survival were recorded, but physiological experiments on brook trout exposed to similar conditions suggest that these fish experienced transient ion loss upon exposure to low pH, followed by ionoregulatory recovery. The proliferation of lamellar chloride cells appears to be related to this acclimation, if one presumes that they are ion conserving in nature.

In the low pH + Al group, a decrease in growth was observed. Consistent with this observation is the increase of white blood cell infiltration of the lymphatic space of the secondary lamellae, evidence of the cytotoxicity of the low pH + Al condition. Again, no physiological measures were performed on fish from this study, but Booth et al. (1988) and Wood et al. (1988) demonstrated ionoregulatory disturbance and decreased oxygen diffusion (based on blood gas measurements) in fish surviving exposure to similar conditions. The increase in diffusion distance is, of course, consistent with measured decreases in oxygen diffusion.

Although the increase of the lamellar dense cells was the most dramatic change in the composition of gill tissue, it is the least understood. The origin and function of these cells need to be investigated further before their proliferation in response to Al exposure at low pH is understood.

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# NOTES

## Hatching Success of Mayfly Eggs at Low pH

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Rowe, L., J. Hudson, and M. Berrill. 1988. Hatching success of mayfly eggs at low pH. Can. J. Fish Aquat. Sci. 45: 1649–1652.

Eggs of the mayflies *Leptophlebia cupida*, *Habrophlebia vibrans*, *Stenonema femoratum*, and *Baetis flavistriga* were reared in soft water at several pH levels (4.0, 4.5, 5.0, and 6.5) in the laboratory. The proportion of eggs undergoing eclosion did not vary with pH. However, in *B. flavistriga*, significant mortality of nymphs occurred before the hatch was complete at pH 4.0 (91%) and 4.5 (12%). Hatching rate of *H. vibrans* was significantly retarded at pH 4.0, 4.5, and especially at pH 5.0, but was unaffected in the other three species. The effects of elevated [$H^+$] on these species are in agreement with their relative acid sensitivity inferred from field surveys.

Les oeufs des éphémères *Leptophlebia cupida*, *Habrophlebia vibrans*, *Stenonema femoratum* et *Baetis flavistriga* ont été élevés en laboratoire dans de l'eau douce à différents pH (4,0, 4,5, 5,0 et 6,5). La proportion d'oeufs qui ont éclos ne variait pas avec le pH. Toutefois, chez *B. flavistriga*, on a observé une importante mortalité des nymphes avant l'éclosion complète lorsque le pH était de 4,0 (91 %) et de 4,5 (12 %). L'éclosion chez *H. vibrans* était significativement retardée à pH 4,0, 4,5 et surtout à pH 5,0, mais elle n'était pas modifiée chez les trois autres espèces. Les effets d'une concentration élevée d'ions $H^+$ chez ces espèces sont conformes à leur sensibilité à l'acide montrée par des études sur le terrain.

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A reduction in abundance and diverity of aquatic insects with decreasing pH has been well documented (Okland and Okland 1988). Fiance (1978) observed a decrease in the recruitment success of the mayfly *Ephemerella funeralis*, following the experimental acidification of a stream, which he attributed to a particular sensitivity of eggs or very early instars. However, little is known about the sensitivity of aquatic insect eggs to elevated concentrations of hydrogen ion ([$H^+$]). Studies of insect egg hatching success at low pH have thus far been limited to species that are known to be acid tolerant. van Frankenhuyzen et al. (1985) found no change in development rate or hatching success in eggs of the caddisfly *Clistoronia magnifica* at pH 6.1 relative to pH 8.1. Similar results were reported for development time and hatching success of four Odonata species at pH 3.5 (Hudson and Berrill 1986). In contrast, mayflies are generally considered sensitive to elevated [$H^+$] (MacKay and Kersey 1985; Okland and Okland 1986), but the sensitivity of the egg stage is unknown.

To test the prediction that mayfly eggs are sensitive to elevated [$H^+$], we measured the proportion of total hatch, the proportion of incomplete hatch, and the hatching rate of four mayfly species in soft water at various pH levels. The selected species (*Leptophlebia cupida*, *Habrophlebia vibrans*, *Stenonema femoratum*, and *Baetis flavistriga*) occur in softwater streams on the Precambrian Shield in the Haliburton region of south-central Ontario and were suspected to have various acid sensitivities.

### *Methods*

*Species and collections* — Synoptic surveys of streams in Haliburton and elsewhere demonstrate that populations of *L. cupida* occur in acidic (pH < 5.0) and less acidic (pH > 5.0) streams whereas *S. femoratum*, *H. vibrans*, and *B. flavistriga* are present only in the latter (Hall and Ide 1987; R. J. Hall, Dorset, Ont., unpubl. data).

With the exception of *S. femoratum*, all eggs used in this study were collected in the field and all four collection sites mentioned below are located in south-central Ontario. *Leptophlebia cupida* that were ovipositing on the surface of Plastic Lake outflow (pH 4.9–5.9; LaZerte 1984) were collected on May 6, 1986, and placed in petri dishes where they then oviposited. *Habrophebia vibrans* were collected in a similar fashion from Costello Creek (pH 6.2–6.5; Hall and Ide 1987) on June 13, 1986. To collect *B. flavistriga* eggs, which are oviposited on underwater surfaces, we placed clean rocks in areas of Jacksons Creek (pH 7.2–8.0) where females had been

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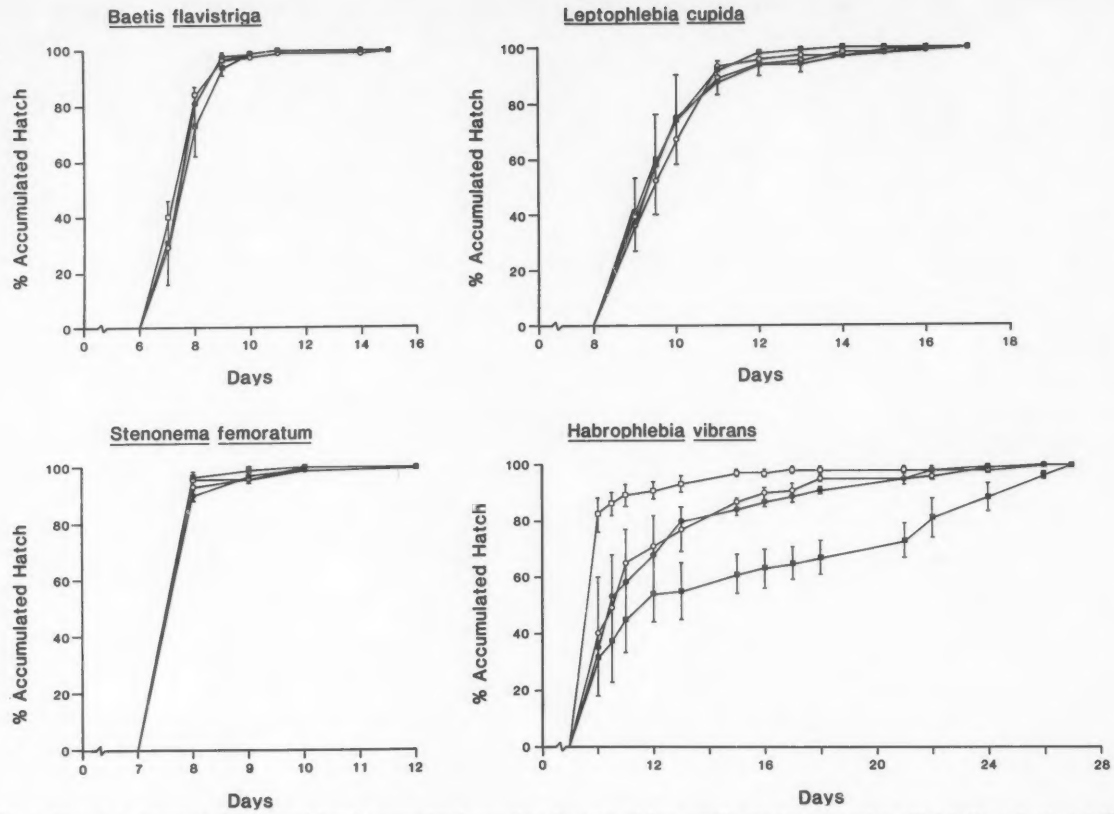


FIG. 1. Mean (± SE) proportion of total hatch accumulated over time for eggs of four mayfly species exposed to pH 4.0 (●), 4.5 (○), 5.0 (■), and 6.5 (□).

TABLE 1. Mean (SE) proportion of total hatch (first row), mean (SE) proportion of total hatch that was incomplete (second row), and total number of eggs exposed at each pH (third row).

| Species | pH 4.0 | pH 4.5 | pH 5.0 | pH 6.5 |
|---|---|---|---|---|
| *L. cupida* | 0.86 (0.036) | 0.90 (0.014) | 0.78 (0.044) | 0.84 (0.046) |
| | 0.00 | 0.00 | 0.00 | 0.00 |
| | 239 | 304 | 233 | 240 |
| *H. vibrans* | 0.89 (0.030) | 0.88 (0.033) | 0.70 (0.107) | 0.89 (0.020) |
| | 0.00 | 0.00 | 0.00 | 0.00 |
| | 481 | 352 | 410 | 388 |
| *S. femoratum* | 0.94 (0.015) | 0.97 (0.006) | 0.94 (0.015) | 0.90 (0.015) |
| | 0.03 (0.016) | 0.01 (0.006) | 0.00 | 0.00 |
| | 258 | 433 | 411 | 437 |
| *B. flavistriga* | 0.80 (0.040) | 0.90 (0.040) | 0.83 (0.042) | 0.85 (0.060) |
| | 0.91 (0.018) | 0.12 (0.013) | 0.01 (0.003) | 0.00 |
| | 478 | 528 | 503 | 613 |

observed ovipositing. The following day (June 23, 1986), ovipositing females and their eggs were collected from these rocks. Final intar *S. femoratum* were collected in late May from Thompsons' Creek (pH 7.2–8.0) and reared to imago in the laboratory. Imagoes were then force copulated (Huff and McCafferty 1974) on May 23, 1986, and these inseminated females were placed in a petri dish to oviposit.

*Exposures* — Water for the exposures was collected from Paint Lake in Haliburton Co., filtered (1 μm), and decarbonated prior to use. The chemical composition (millimolar) of this water was $Ca^{2+}$ = 0.10, $Na^{+}$ = 0.10, $Cl^{-}$ = 0.09, $K^{+}$ = 0.02, $Mg^{2+}$ = 0.06, $SO_4^{2-}$ = 0.08, and Al = 0.001. Experimental treatments were pH 4.0, 4.5, 5.0, and 6.5 which were established and maintained with 0.1 M $H_2SO_4$ and 0.1 M

KOH. pH varied no more than 0.2 unit over the exposure period. Exposures were conducted in modified petri dishes partially submerged in 300-mL fingerbowls (J. Hudson, unpubl. data). The petri dish was fitted with a Plexiglas chamber that fills with water through an egg-retaining mesh at its base. Eggs rested on this 20-μm nitex mesh so that aerated water from the fingerbowl could circulate over them. Four replicates at each pH were used, for a total of 16 fingerbowls for each species. The exposures were conducted at room temperature (21–26°C) under 15 h/d incandescent light.

Exposures of eggs began within 32 h of collection and all exposures for a given species were initiated on the same day. Eggs from at least three females of each species were pooled, mixed, and placed in treatments in quantities varying from 61 to 179 per replicate. Fingerbowls were monitored for hatches and pH at least once every 2 d and more often during times of rapid hatching. Hatched eggs and their nymphs were removed and discarded.

Three questions were addressed: does exposure of eggs to elevated $[H^+]$ affect the proportion of total hatch, the proportion of incomplete hatches, and the rate at which eggs hatch. Total hatch was considered as the proportion of eggs that had undergone eclosion, characterized by a longitudinal fissure in the chorion. In some cases, nymphs died before freeing themselves from this fissure; in such cases the hatch was considered incomplete. Because of possible container (petri dish) effects, observations on the timing of individual egg hatches could not be treated as independent data. As an independent measure of hatching rate, the proportions of total hatch were summed over the duration of the experiment. This method yields one datum per container that describes the area under each hatch rate curve (Fig. 1). A rapid hatch rate would have a high sum (e.g. over a 4-d experiment, $0 + 0.75 + 0.95 + 1.0 = 2.7$) relative to a slower hatch rate ($0 + 0.25 + 0.75 + 1.0 = 2.0$). For all three variables described above, the differences between treatments were assessed using a nonparametric analysis of variance test (Kruskal–Wallis).

### *Results and Discussion*

No significant difference ($p > 0.1$) in the proportion of total hatch (eclosion) occurred across pH treatments in any of the four species (Table 1). However, the proportion of incomplete hatches was significantly increased ($p < 0.001$) at low pH in *B. flavistriga* (Table 1). Incomplete hatches accounted for 91 and 12% of total hatch at pH 4.0 and 4.5, respectively. Rare and insignificant incomplete hatches were also observed at low pH in *S. femoratum* (3% at pH 4.0; 1% at pH 4.5). No incomplete hatch occurred in these species at pH 6.5 or in either *L. cupida* or *H. vibrans* at any pH. Nymphs from these incomplete hatches were assumed dead; their body had discolored to an opaque white and had not completely freed themselves from the shell. A similar inability of larvae to free themselves from hatched eggs has been observed in acid-stressed Atlantic salmon (*Salmo salar*) (Daye and Garside 1977).

Hatching rates of *L. cupida*, *S. femoratum*, and *B. flavistriga* were not significantly different across treatments ($p > 0.05$). However, *H. vibrans* responded unusually. Although total hatch remained unaffected by exposure to pH < 6.5, it was delayed by exposure to pH 4.0, 4.5, and particularly pH 5.0 ($p < 0.01$). The greater sensitivity to pH 5.0 remains unexplained. *Habrophebia vibrans* had the longest hatching period at all pH's of these four species and therefore the longest exposure to elevated $[H^+]$. The temperatures at which these exposures were conducted (21–26°C) are higher than those to which eggs would be exposed in the field. Since development rates of mayfly eggs decrease at low temperature (Howe 1967), the egg stage would be of a longer duration in the field than those reported here. Therefore, it is possible that hatching rates of all of these species may be affected by exposure to elevated $[H^+]$ at the lower field temperatures. Decreased hatch rates may result from either decreaed embryonic development rates or impairment of the hatching enzyme, as has been suggested in other taxa (Peterson et al. 1980; Freda 1986).

The deleterious effects of exposure to elevated $[H^+]$, observed here on the egg stages of *B. flavistriga* and *H. vibrans*, are in agreement with the acid sensitivity of these species and genera inferred from field surveys (Hall and Ide 1987; MacKay and Kersey 1985; Raddum and Fjellheim 1984). While we did not attempt to monitor the mortality rates of nymphs following complete hatch, the occurrence of incomplete hatching at low pH may reflect a particular sensitivity of early instar nymphs (Fiance 1978; Allard and Moreau 1987). *Stenonema femoratum* is, according to field surveys (R. J. Hall, unpubl. data), a relatively sensitive species; however, despite the small number of incomplete hatches, our data do not indicate sensitivity, and later stage nymphs also survive exposure to low pH (Rowe 1987). Further laboratory studies comparing early with later stages are required to determine whether early stages of aquatic insects are relatively sensitive to elevated $[H^+]$.

In conclusion, these results with mayflies add to a growing list of taxa where the egg stage is impaired at realistic low pH levels. Effects of $H^+$ on development or hatching of eggs have also been demonstrated at pH 4.0–5.5 in fish (Peterson et al. 1980), pH 3.5–5.0 in amphibians (Freda 1986), pH 5.0–5.5 in molluscans (Servos et al. 1985), and pH 4.5–5.5 in crayfish (Berrill et al. 1985).

Future research with acid-sensitive insects should therefore include the egg stage and early instars. Experiments including increased aluminum concentrations, common to acid streams, may also demonstrate greater $H^+$ toxicity at higher pH levels.

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# Computer-Driven Motor Control with Positional Memory for Mechanical Microscope Stage

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A computerized control system for the *X–Y* mechanical stage of an inverted microscope was constructed using relatively inexpensive electrical components and requiring minimal modification to the stage. The control consists of two linear stepper motors, one for each axis, driven by commands from an IBM-compatible PC via a serial communications port and a simply constructed electrical controller. Menu-driven software regulates the motions of the steppers and allows keyboard input of data on the type and number of particles in settling chambers or on microscope slides. Frame locations of particles are stored automatically to facilitate reexamination of samples. The system allows samples to be removed and replaced while retaining the ability to relocate particles of interest.

On a construit un système de commande informatisé pour le platine à chariot selon les axes *X–Y* d'un microscope inversé en utilisant des composantes électriques relativement peu coûteuses et n'exigeant qu'une modification minime au platine. Le système se compose de deux moteurs pas-à-pas linéaires, un pour chaque axe, actionnés par des commandes provenant d'un ordinateur personnel compatible avec IBM via une porte de communications en série et un régulateur électrique de construction simple. Un logiciel piloté par menu règle les mouvements des moteurs pas-à-pas et permet l'introduction par clavier des données sur le type et le nombre de particules dans les chambres de sédimentation ou sur les lames de microscope. La localisation des particules dans le cadre est emmagasinée automatiquement pour faciliter le réexamen des échantillons. Le système permet d'enlever les échantillons et de les remplacer tout en permettant de relocaliser les particules présentant un intérêt.

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Common methods for determining the taxonomic composition and abundance of microscopic organisms such as plankton, bacteria, microfauna, and meiofauna involve scanning samples in settling chambers or mounted on slides. It is often necessary to review samples after counting to confirm identifications, measure body dimensions, etc. Furthermore, if identifications or measurements are made while counting, items near the edges of the fields of view must be moved into better position, magnification changed, etc., before resuming counting. For these and other reasons, microscopic examination of such samples can be tedious, time consuming, and error prone. Therefore, we investigated the possibility of using a computerized, motor-driven microscope stage with automated motion control and acquisition of data on counts and locations of organisms.

Commercial microscope stage control systems are available but we found them to be inordinately expensive and often unsuitable for our purposes. Of several noncommercial systems in the literature, one proposed by Capowski and Schneider (1985) was the most interesting but lacked position feedback. A stage control design developed at the National Research Council of Canada's Atlantic Regional Laboratory for use on a scanning electron microscope (Dr. Paul Odense, pers. comm.) was also of interest but the computer is only used to store positions, not to control the stage. A joystick is used for this purpose. Since we could find no reasonably priced commercial or noncommercial system which satisfactorily met our needs, we opted to construct the system which we present here.

*Mechanical Design*

The basic components of the stage control are two linear stepper motors (AIRPAX L92211-P2), each capable of producing up to 11 N, the force required to move the stage of the microscope which we used, a Zeiss ICM 405. Each motor produces a linear motion in a shaft which in turn moves the stage along the *X*- or *Y*-axis. The movement occurs in discrete steps of 25 μm, sufficiently small for our purposes. Figure 1 illustrates the configuration of the motors on the *X*- and *Y*-axes of the stage. The motor shafts are threaded into brackets mounted on the stage. They can be disengaged easily from the stage if manual control of the stage is desired. Other arrangements were considered but the one chosen requires no gearing, no intermediate connections, and no modifications to the mechanical stage other than mounting the brackets. The present configuration does not interfere with any uses of the microscope that do not require the motor-driven stage.

The major problem encountered in using these stepper motors is that there is some slack in the motion of the shaft which, if not corrected, would cause inaccurate positioning when the direction of motion is changed. We have overcome this problem by always approaching a position from one direction only in each axis. Thus, when stepping, for example, from left to right the motor approaches the position directly from the left, but when stepping in the opposite direction (right to left) the motor is programmed to overshoot to the left by a sufficient number of steps (four or five) to compensate for the slack and then to backstep onto position from the left. The motors can step at a rate of 250 steps per second, so the time delay is insignificant.

*Electrical Design*

Our main concerns in producing an electrical design for the stage control system were to keep total costs low and to provide the user with an easily used and flexible system which would have good positional integrity. The linear stepper motors were run using a 12-V D.C. supply, and AIRPAX SAA 1027 driver chips were used to control the stepping of the motors' phases.

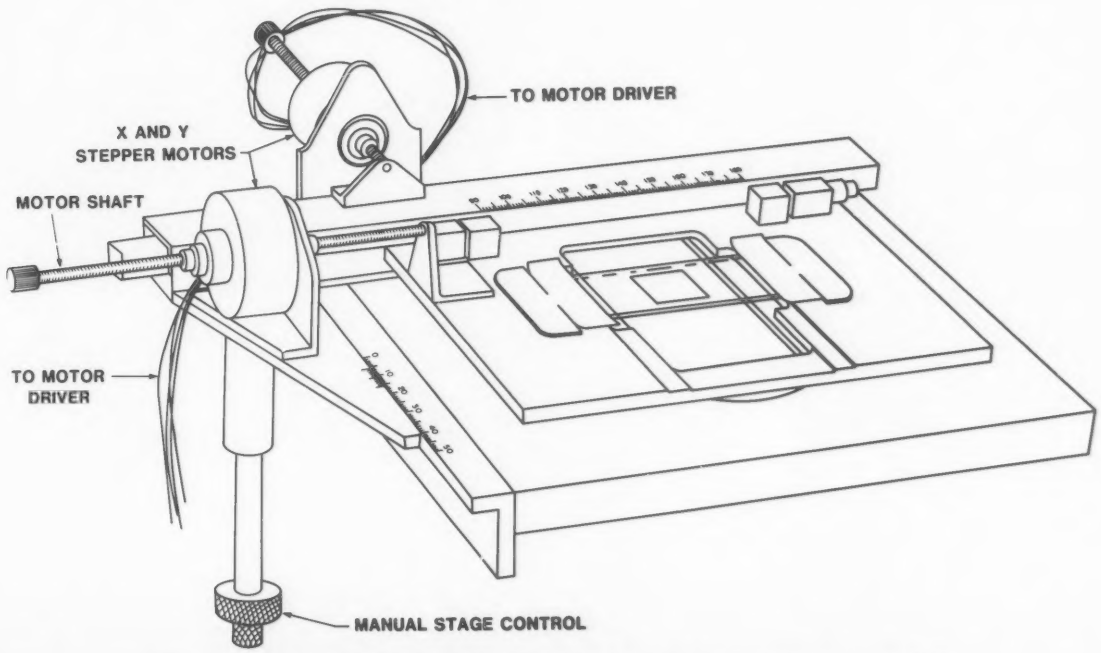


FIG. 1. Mechanical stage of Zeiss ICM405 inverted microscope with linear stepper motors attached.

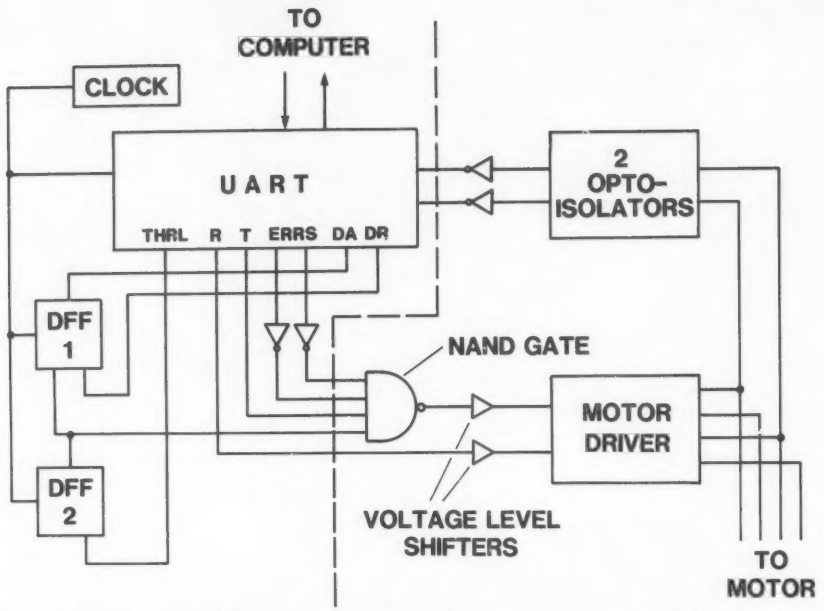


FIG. 2. Simplified circuit diagram of the stepper motor control unit. The right side of the figure shows the circuit for a single motor only. Lines leading from the UART are labelled as follows: R, receive; T, transmit; ERRS, parity and framing errors; DA, data available; DR, data available reset; THRL, transmitter holding-register load (initiates return of motor phase information to computer).

The motors are capable of 250 steps per second at maximum load, so if the communications port is set for 8 data bits with 1 start and stop bit, then the baud rate would have to be 2500 baud to maximize their capability. This is well within the PC's maximum of 9600 baud. The design problem was then narrowed down to devising a circuit that would interface the driver chips to our Panasonic Exec. Partner, an IBM-PC compatible computer. The Exec. Partner has both parallel and serial interfaces. We selected serial communications because the complexity of interface connections is greatly reduced if the serial port is used.

A universal asynchronous receiver–transmitter (UART) with simple control hardware was used to instruct the motors to step whenever a command was sent by the computer. The UART method was chosen in preference to using an independent microcontroller because electronic costs would be reduced using the UART system, and more importantly, all of the control of the motors is placed in the computer software where it can be easily accessed and modified.

The control circuit was designed to take 2 bits of each 8-bit serial word to step a motor. One bit selects the direction and the other instructs the motor to step. Up to four motors could be controlled simultaneously with an 8-bit word, so in addition to the *X* and *Y* position, such things as focus and lighting intensity could also be controlled.

The commercially available stage control systems which we investigated lacked feedback from the motors to tell the computer if they had stepped as instructed and thus keep track of the correct position of the stage. There are several possible methods for reporting the position of the microscope stage, most of which would add significantly to the cost of the system and would involve substantial modifications to the microscope stage. The feedback method which we have incorporated into our system makes use of the fact that the four motor windings make a complete on/off cycle every four steps of the motor. By monitoring the lines and feeding the information back to the computer, a control circuit can be based on the current phase of each motor and will indicate whether a step command has been received and carried out by each motor. This involves no modifications to the stage and minimal cost in electronic components.

For a given series of step commands, the motor windings will cycle repeatedly through four discrete phases:

| Phase | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| Step 1 | 0 | 1 | 0 | 1 |
| Step 2 | 0 | 0 | 1 | 1 |
| Step 3 | 1 | 0 | 1 | 0 |
| Step 4 | 1 | 1 | 0 | 0 |

where a 1 indicates 12 V applied to a winding. The steps can be monitored by looking at phases 1 and 3 only to give the following binary pattern:

| Phase 1 | Phase 3 | State in cycle |
|---|---|---|
| 0 | 0 | 0 |
| 0 | 1 | 1 |
| 1 | 0 | 2 |
| 1 | 1 | 3 |

Figure 2 is a simplified schematic of the motor control and feedback circuit. If no transmission errors are detected on the serial lines, i.e. framing or parity errors, the first flip-flop (D-type, DFF1 in Fig. 2) sends a step pulse out to the appropriate

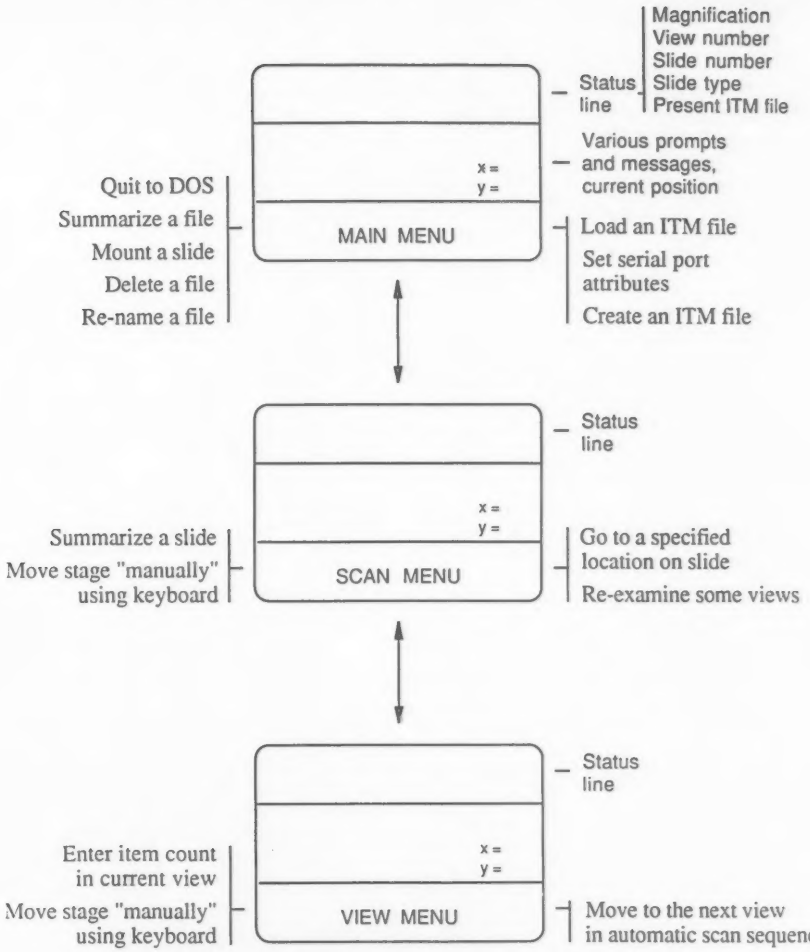


FIG. 3. Flowchart showing on-screen menus for operating the stage control system.

(*X*- or *Y*-axis) motor-driver chip through a NAND gate (error check) and a 5- to 12-V voltage-level shifter. The driver chip steps the motor and the voltage level is returned to the UART via the associated (*X*- or *Y*-axis) opto-isolator to confirm motion and position. The opto-isolators are used in the feedback loop to protect the UART from high currents generated when the motors are stepped and to convert the 12-V signal to 5 V for the UART. After one UART clock cycle, a second flip-flop (DFF2) toggles and instructs the UART to send data back to the computer. The system then waits for the next step command.

### *Software*

The software that controls the stepping motors has been designed to automate stage movement functions such as scanning in transects and moving to fields of interest on microscopic preparations. It has been written to accomodate round and rectangular scan areas. In addition, the software is an accounting system wherein data on locations and counts (the latter entered by the user) of many (up to 100 as presently written) different types of particles or structures may be stored in disk files. In our system, the microscope is video-coupled to a Kontron MOP-40 digitizing tablet with video overlay; however, the stage control system is completely independent of the Kontron system. We have defined the size of the field of view by the active region outlined by the Kontron video overlay using a given microscope magnification. The edges of the active region are well within the video field of view, so particles near the edges are easily visible. The number of motor steps required to move to an adjacent field of view in either the *X* or *Y* direction using given magnification is specified in the stage control software. Both view size and number of motor steps per view are easily modified within the program for uses not involving the video overlay (for example, using an ocular grid).

The main program and five "include" files (procedure programs) are written in Turbo Pascal, Version 3.0 (Borland International 1985). Turbo Asynch, a commercially available package (Blaise Computing Inc. 1985), is used to manage the serial communications including opening and closing a port, setting port attributes, and reading/writing to and from a port. The total package, excluding source code, occupies 85K bytes of disk space.

The user is presented initially with a Main Menu, followed, as requested, by a Scan Menu and a View Menu. Figure 3 is a

flow chart which outlines the features of the three menus. When one of the procedures is chosen, a roll-down menu appears with more detailed information and prompts. The software is written to allow the user maximum flexibility and yet provide very clear instructions for each operation. All commands from the user are entered via the keyboard because this does not require the eyes to be averted from the microscope image for many functions. However, a mouse or joystick could be used if desired.

The following is a typical procedure followed to examine a slide preparation for the first time:

(1) Enter LCOM (LCOM.EXE loads assembler routines for Turbo Asynch) and then SCAN (SCAN.COM is the main program, 53K bytes in size). The Main Menu appears.

(2) Place the slide or settling chamber on the stage and enter MOUNT (from the Main Menu). Enter an identification number for the data file to be created for the slide (a text file, named SLIDE.xxx where xxx is the identification number of the current slide). Using keyboard motion control, locate a well-defined point of origin on the sample such as one of the coverslip corners or an *X* or *Y* tangent to the outer circle of a round coverslip or settling chamber. This position is recorded in the data file by entering the appropriate command from the MOUNT menu. The MOUNT procedure requires that the positions of this and two other points on the perimeter of the area to be scanned are recorded so fields of interest can be relocated precisely. If a standard 2.5-cm-diameter settling chamber is used, only the single point of origin is required. At this time, a text file (name.ITM) can be created or loaded to specify a list of up to 100 different particle types or features, defined by the user, whose locations and numbers will be stored in the data file.

(3) Enter the Scan Menu and start scanning the sample. Each time the "space bar" on the keyboard is pressed, the stage is moved to an adjacent field of view along transect lines whose widths are set by the user for any given magnification. The user can also depart from the transect pattern to view a field of interest more closely by using keyboard directional commands and then return to within less than 2.5 μm of the point of departure. The transect can then be continued.

(4) The View Menu allows the user to enter the numbers of particles or features in the present field of view. The location is automatically stored along with this information.

(5) The same sample may be removed and later replaced on the stage (in the same position) and the SLIDE.xxx file associated with it may be called up from the Main Menu to reexamine specific fields of interest or to continue scanning where it was left off. Settling chambers may be removed and replaced in the same position by using a pin on the stage plate which fits into a notch on the perimeter of the chamber base. Moving of settling chambers must be done with care because some types of settled particles can be very mobile.

## *Conclusion*

The stage control system is flexible enough to be used in many applications involving scanning of microscopic preparations. The software has been written for an IBM-compatible PC and most modifications involve only the software, which is clearly documented. The simplicity of the motor mount design also means that it can be easily adapted to microscopes other than the Zeiss ICM405. If steps smaller than 25 μm are required, the controller board may be used as is and only the motors need be changed, provided they are compatible with the Airpax driver chips. This would increase the cost of the present system (under $600 Canadian, exclusive of the PC and labor). Nonetheless, the system would still be inexpensive relative to commercially available stage controls while incorporating feedback for greater reliability, a feature few other systems offer.

Detailed schematics of the electronics, user instructions, and copies of the compiled software and PASCAL source code are available on request from the corresponding author. Please include a 5 ¼-in. floppy disk with the request.

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# PERSPECTIVES

# Larval Size and Recruitment Mechanisms in Fishes: Toward a Conceptual Framework[1]

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Understanding the mechanisms controlling recruitment in fishes is a major problem in fisheries science. Although the literature on recruitment mechanisms is large and growing rapidly, it is primarily species specific. There is no conceptual framework to integrate the existing information on larval fish ecology and its relationship to survival and recruitment. In this paper, we propose an integrating framework based on body size. Although all larval fish are small relative to adult fish, total length at hatching differs among species by an order of magnitude. As many of the factors critical to larval survival and growth are size dependent, substantially different expectations arise about which mechanisms might be most important to recruitment success. We examined the evidence for the importance of size to feeding and starvation, to activity and searching ability, and to risk of predation. Regressions based on data from 72 species of marine and freshwater species suggest that body size is an important factor that unifies many of the published observations. A conceptual framework based on body size has the potential to provide a useful integration of the available data on larval growth and survival and a focus for future studies of recruitment dynamics.

La compréhension des mécanismes régissant le recrutement des poissons est un sujet important de l'halieutique. Bien que l'on dispose d'un grand nombre de documents sur les mécanismes du recrutement et que ceux-ci augmentent rapidement, il s'agit surtout d'un phénomène spécifique d'espèce. Il n'existe pas de cadre conceptuel pour intégrer les données existantes sur l'écologie des larves de poisson et son rapport avec la survie et le recrutement. Dans le présent article, nous proposons un cadre intégré fondé sur la taille. Bien que toutes les larves soient petites par rapport à l'adulte, la longueur totale à l'éclosion diffère d'un ordre de grandeur entre les espèces. Étant donné que plusieurs des facteurs critiques pour la survie des larves et leur croissance sont fonction de la taille, des hypothèses très différentes sont formulées relativement aux mécanismes qui pourraient être très importants pour le succès du recrutement. Nous avons étudié les données relatives à l'importance de la taille par rapport à l'alimentation et à l'inanition, à l'activité et à l'aptitude à chercher la nourriture, et le risque de prédation. D'après des régressions fondées sur des données provenant de 72 espèces de poissons marins et dulçaquicoles, la taille est un facteur important qui constitue un point commun dans plusieurs observations publiées. Un cadre conceptuel fondé sur la taille peut permettre une intégration utile des données disponibles sur la croissance et la survie des larves et constitue un sujet en vue d'autres études sur la dynamique du recrutement.

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Interest in larval fishes and factors influencing recruitment variation has increased rapidly in recent decades (Blaxter 1974; Lasker and Sherman 1981). A field that had been dominated by a seemingly endless search for the "stock–recruitment" relationship has focused increasing effort on understanding *mechanisms* underlying recruitment, whether they be largely driven by environmental variation or biotic interactions. Recent interest in earlier life history stages was stimulated by the need to understand sources of variation in the adult population size of exploited species. All larval fish are small relative to the size of adults, producing the perception that they will have similar ecological responses to important abiotic or biotic variables. Yet a careful analysis of the literature quickly reveals that larvae of different species differ widely in their response to these variables.

Historically, most studies of factors affecting larval fish survival and subsequent recruitment to the adult population have taken a single-species approach. In general, attempts to extend the results of such species-specific research to other larval fishes have met with limited success because these studies often reached conflicting conclusions regarding the key factors gov-

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erning survival. For instance, some species appear highly vulnerable to starvation (Lasker 1975, 1978), while others are more likely controlled by predation or abiotic factors (Lett and Kohler 1976; Winters 1976; Hunter 1981; Bailey and Houde 1988).

Body size has long been recognized as an important variable influencing many aspects of the physiology, ecology, and behavior of living organisms (Thompson 1917; McMahon and Bonner 1983; Peters 1983; Calder 1984). While all larval fish are small, length at hatching varies among species by over an order of magnitude. Thus, initial weight may vary by three or more orders of magnitude. Moreover, while many fish increase in weight by more than five orders of magnitude over their life span, three orders of magnitude of this change may occur in the first year of life (Werner and Gilliam 1984; Houde 1987). If one were to allocate research effort to fishes scaled by weight stanzas (physiological time) rather than by years (calendar time), one would spend much less time on large adults (where body size does not change much) and would concentrate on dynamics in the first year of life.

If size-dependent processes are important, one should expect substantial differences in larval life histories based on differences among species in body size at hatching. Many factors influencing the behavior and physiology of larval fish are likely to be size dependent (Hunter 1981). Small larvae may be more susceptible to starvation because of the limited energy reserves in their yolk sac (Hunter 1981). In addition, small larvae may have short reactive distances and limited swimming abilities which will limit their searching ability (Blaxer 1986; Webb and Weihs 1986). The size of food particles ingested depends on both mouth gape and the ability to capture the particle, both of which clearly increase with larval size (Hunter 1981). Body size will also influence vulnerability to predators through differential encounter rates, escape ability, and predator gape limitation (Blaxter 1986; Zaret 1980). The effects of size-dependent processes acting on larval fishes may be profound. They must be considered comprehensively and the effects of various size-dependent mechanisms must be integrated to fully understand their role in survival and subsequent recruitment variability.

Generally, the implications of body size have been well investigated for larger juvenile and adult fishes. But these size-dependent relationships have not been thoroughly elaborated for larval fishes. The purpose of this paper is to examine the empirical evidence for the importance of size-dependent processes to the ecology of larval fish and to establish a conceptual framework that will integrate the available species-specific observations into a more general perspective. In this way, size-dependent ecological characteristics of particular species may be placed in a larger context, and work on the larval ecology of little studied species may profit more directly from previous research.

## Methods

To start our investigation of the influence of body size on larval survival and subsequent recruitment, we hypothesized functional relationships between a number of life history traits and body length at hatching or at later points in the larval or juvenile stage. The traits we chose can be divided into four main categories related to feeding, activity, vision, and predation risk (Table 1). While all of the traits influence subsequent recruitment, for many the effect is indirect, acting through larval growth rate to influence the probability of larval survival.

To assess our proposed hypotheses, we surveyed the literature for relevant data, focusing our effort on papers published in the last 5 yr. We examined fisheries and aquatic journals as well as previously published reviews (Hunter 1981; Blaxter and Hunter 1982; Blaxter 1986). We only included studies on species that do not show significant parental care after hatching.

Total length was used as our measure of body size. Some of the traits we examined are probably more closely tied to weight than length, but weight is more difficult to measure and was rarely reported. We recognize that temperature is likely to affect some of these traits, particularly those related to metabolic rate. Unfortunately, the reviewed experiments were usually done at "typical" temperatures for the species being studied, rather than over a range of temperatures. Consequently, it was impossible to account for temperature effects using ANOVA techniques. For appropriate variables, we addressed the influence of temperature by repeating our analysis after standardizing results to 15°C using $Q_{10} = 2.3$ (Checkley 1984). This approach provides some indication of whether the size effects persist, independent of temperature. Other variables, including methodological differences among studies, are likely to contribute to variance in size-dependent relationships. However, as an initial step, using total length as a measure of size should be adequate to identify the generalities we seek.

We found information on 72 species of marine (54), freshwater (16), and anadromous (4) fishes from 30 different families (Table 2). The data included here are derived from 128 references out of more than 500 we examined.[2] The examined species provide a wide range of body size at hatching, thus enhancing our ability to detect size-related patterns in the data. Our analysis includes studies on species with egg diameters ranging from 0.8 to 9.7 mm (Fig. 1a) and length at hatching ranging from 1.6 to 17.6 mm (Fig. 1b). Although portions of the literature have previously been reviewed (Hunter 1981; Blaxter and Hunter 1982; Blaxter 1986; Webb and Weihs 1986), it has not been reviewed to examine a broad set of a priori hypotheses regarding the mechanisms controlling recruitment.

Criteria for inclusion of data from a particular species for each trait were decided prior to the review. Where possible, the original literature reference was used instead of later reviews. In most cases, data were derived from tables given in the original paper, but in some cases, data were interpolated from graphs. No single reference provided data on all traits for a particular species. If no larval hatching size was given in a reference, we substituted an average value from other references for that species. These averages were calculated from values given in as many references as possible. All other data were included as the original authors presented them; we did not judge the data or sort the results according to methods or "quality." All data were converted to standard units (Table 1). The data were analysed for the degree of fit to the hypothesized relationships using either a linear or simple exponential function. We report the function giving the best fit. In all cases the value of "$n$" quoted with the regression equation is the number of independent observations used by the analysis. Calculations were done using Statistical Analysis System (SAS Corporation, Cary, NC) at the Triangle Universities Computing Center.

[2]A complete listing of the original references used in this review is provided in the Appendix. The data used to develop the regression relationships given below are available, at a nominal charge, from the Depository of Unpublished Data, CISTI, National Research Council of Canada, Ottawa, Canada K1A 0S2.

TABLE 1. Larval traits considered in this analysis, their definitions, and units of measurement.

| Trait (units) | Definition |
|---|---|
| Yolk absorption (d) | Time taken to fully absorb the yolk from hatching |
| Point of no return (d) | Time after which the effects of starvation are irreversible, i.e. even if feeding is resumed, the larva still dies. It is defined for an individual, but can only be measured for a group |
| First possible feeding (d) | Time at which a larva is first physically or anatomically capable of feeding |
| First typical feeding (d) | Time at which larvae usually feed for the first time, defined for a group |
| Time to 50% mortality (d) | Time taken for half of the larvae in a group to die when starved |
| Sustained swim speed (cm/s) | Speed at which fish swim for extended period of time |
| Burst swim speed (cm/s) | Speed attained in short sprints, usually during prey capture attempts or in predator avoidance |
| Reactive distance (mm) | Maximum distance at which a potential prey item is recognized as such |
| Search volume (L/h) | Function of sustained swim speed and reactive distance: equivalent to the volume of water searched per unit time |
| % capture success | Percent of all "observed" capture attempts that were successful |

## Results

For each larval trait (Table 1), we first present our a priori hypothesis for its relationship with larval length and then the data from the literature to evaluate the hypothesis. Within each category, some traits relate directly to larval size at hatching, while others are general functions of body length for post-hatching larvae and juveniles.

### Feeding and Larval Size

Initially, a larva obtains nutrition from its yolk, and later from food in the environment. To avoid starvation, a larva must be able to feed before its yolk supply is depleted. Accordingly, the importance of starvation in early life history depends upon the traits that govern access to both endogenous and exogenous sources of food.

Egg size sets a limit on the size of larva that can hatch from it (length at hatch = 1.96 + 1.89 egg diameter, $r^2 = 0.40$, $p < 0.0001$, $n = 100$). Within this limit, the partitioning of resources between larva and yolk supply may vary (Blaxter and Hempel 1963). An index of the amount of yolk available to a larva, relative to its energy demands, is the time to absorption of the yolk supply. The time to yolk absorption depends upon yolk volume, which is an exponential function of yolk sac diameter. Therefore, we hypothesized the time to yolk absorption to be an accelerating function of hatching length.

Time to yolk absorption did increase with larval length at hatching. Contrary to expectation, a linear model gave the best fit (Fig. 2). Times to yolk absorption varied from slightly longer than 1 d in 2.9-mm bay anchovy (*Anchoa mitchilli*) (Houde 1974) to 20 d in 9.9-mm *Coregonus wartmanni* (Braum 1967). Times to yolk absorption for marine, freshwater,and anadromous species were not significantly different (demonstrated by a nonsignificant habitat × length interaction, $F_{2,82} = 2.65$, $p = 0.079$), although the freshwater species we examined did tend to have longer times to yolk absorption. When standardized to 15°C to remove potential temperature effects, the strength of the size-dependent relationship increased, accounting for an additional 7% of the variation. This suggests a robust size-dependent relationship. The greater yolk supply of a large larva affords it greater flexibility in when it can shift to exogenous food than does the limited yolk of a small larva, even when the temperature differences are accounted for.

No larva can feed before it is functionally capable (i.e. its mouth and digestive system must have developed), but it must feed prior to reaching irreversible starvation or a "point of no return" (PNR). These limits define the period within which first feeding must occur if the larva is to survive. To understand size-dependent patterns in larval susceptibilities to starvation, one must understand how size influences first possible feeding and PNR. To assess these patterns, we proposed three hypotheses, summarized in Fig. 3.

*Hypothesis 1*

As large larvae are generally more developed at hatching than small larvae, we proposed that time to first possible feeding would be a negative function of larval size at hatching.

*Hypothesis 2*

Large larvae have more yolk and a larger body mass than small larvae. Therefore, we hypothesized that time to PNR would be a positive function of body length at hatching. Because PNR is likely related to the volume of the larva's energy reserves, we proposed an accelerating function.

*Hypothesis 3*

The difference between time to PNR and first possible feeding is an index of the flexibility allowed in timing of first feeding. In combination, the two previous hypotheses suggest that larvae hatching at large sizes experience a greater "window of opportunity" in which to first feed than larvae hatching at small sizes.

Hypothesis 1 was supported by the data, although the relationship is rather noisy (Fig. 4). Time to first possible feeding

TABLE 2. Families and species of marine, freshwater, and anadromous fishes used in this study (listings are according to habits reported in the studies used). Numbers refer to references (Appendix) used to provide data for the analysis.

*Marine*

| | |
|---|---|
| Ammodytidae | |
| *Ammodytes hexapterus* | 92 |
| Atherinidae | |
| *Atherina presbyter*, sandsmelt | 90 |
| *Leuresthes tenuis*, grunion | 81 |
| Belonidae | |
| *Belone belone*, garfish | 102 |
| Blenniidae | |
| *Coryphoblennius galerita*, Montagu's blenny | 30 |
| Bothidae | |
| *Paralichthys olivaceus* | 55 |
| *Scopthalmus maximus*, turbot | 4, 8, 124 |
| Carangidae | |
| *Trachurus symmetricus*, jack mackerel | 2, 5, 113, 114, 115 |
| Clupeidae | |
| *Alosa sappidissima*, American shad | 58, 109 |
| *Brevoortia patronus*, gulf menhaden | 38 |
| *Brevoortia tyrannus*, Atlantic menhaden | 37, 38 |
| *Clupea harengus*, herring | 9, 10, 12, 13, 76, 77, 83, 103, 108, 115, 116, 123 |
| *Harengula pensacolae*, scaled sardine | 47 |
| *Sardina pilchardus*, pilchard | 11, 13, 104 |
| *Sardinops caerula*, Pacific pilchard | 1, 60, 65 |
| Cottidae | |
| *Ascelichthus rhodorus*, rosylip sculpin | 79 |
| Cyprinodontidae | |
| *Fundulus heteroclitus*, mummichog | 58, 95, 109 |
| Engraulidae | |
| *Anchoa mitchilli*, bay anchovy | 45, 48, 58 |
| *Engraulis capensis*, cape anchovy | 17 |
| *Engraulis japonica*, japanese anchovy | 33 |
| *Engraulis mordax*, northern anchovy | 31, 51, 52, 54, 63, 66, 67, 84, 89, 115, 121, 122 |
| *Engraulis ringens*, Peruvian anchoveta | 57, 101, 119 |
| Gadidae | |
| *Gadus morhua*, Atlantic cod | 8, 9, 61, 70, 115 |
| *Merlangius melangus*, whiting | 40 |
| *Melanogrammus aeglefinus*, haddock | 68, 70 |
| *Merluccius productus*, Pacific hake | 3, 7 |
| *Theragra chalcogramma*, walleye pollock | 88 |
| Haemulidae | |
| *Haemulon plumieri*, white grunt | 107 |
| Labridae | |
| *Lachnolaimus maximus*, hogfish | 20 |
| Lophiidae | |
| *Lophius piscatorius*, angler | 71 |
| Percichthyidae | |
| *Morone saxatilis*, striped bass | 58 |
| Pleuronectidae | |
| *Embassichthys bathybius*, deepsea sole | 98 |
| *Hippoglossoides platessoides*, American plaice | 50 |
| *Isopsetta isolepis*, butter sole | 99 |
| *Limanda ferragunea*, yellow tailed flounder | 21, 49, 127 |
| *Limanda limanda*, dab | 87 |
| *Parophrys vetulus*, English sole | 64 |
| *Platichthys flesus*, flounder | 10 |
| *Pleuronectes platessa*, European plaice | 8, 9, 13, 82, 105, 106, 126 |
| *Pseudopleuronectes americanus*, winter flounder | 18, 69 |
| Sciaenidae | |
| *Genyonemus luneatus*, white croaker | 120 |
| *Leiostomus xanthurus*, spot | 93, 94 |
| *Sciaenops ocellatus*, red drum | 74 |

TABLE 2. (*Concluded*)

| | |
|---|---|
| Scombridae | |
| *Scomber japonicus*, Pacific mackerel | 53, 62 |
| Serranidae | |
| *Paralabrax clathratus*, kelp bass | 19 |
| *Paralabrax maculatofasciatus*, barred sand bass | 19 |
| *Paralabrax nebulifer*, spotted sand bass | 19 |
| Soleidae | |
| *Achirus lineatus*, lined sole | 45, 48 |
| *Solea solea*, sole | 13, 124 |
| Sparidae | |
| *Archosargus rhomboidalis*, sea bream | 45, 46, 48, 111 |
| *Lithognathus mormyrus* | 27 |
| *Sparus aurata*, gilthead | 128 |
| *Stenotomus chrysops*, scup | 34 |
| Sphyraenidae | |
| *Sphyraena borealis*, northern sennet | 44 |
| *Freshwater* | |
| Centrarchidae | |
| *Lepomis macrochirus*, bluegill | 110, 117 |
| Clupeidae | |
| *Alosa pseudoharengus*, alewife | 36, 58, 59, 96, 109 |
| Cyprinidae | |
| *Aspidoparia morar*, Indian carp | 78 |
| *Danio rerio*, zebra danio | 32 |
| *Leuciscus leuciscus*, dace | 85, 124 |
| Percidae | |
| *Perca flavescens*, yellow perch | 23, 42, 43, 110 |
| *Stizostedion canadense*, sauger | 86 |
| *Stizostedion vitreum vitreum*, walleye | 43, 58, 75, 80, 86 |
| Salmonidae | |
| *Coregonus artedii*, lake herring | 23, 39, 56, 109, 124 |
| *Coregonus clupeaformis*, lake whitefish | 15, 23, 41, 109, 112 |
| *Coregonus hoyi*, bloater | 15, 97 |
| *Coregonus laverutus*, Lake Syamozero whitefish | 14, 15, 24 |
| *Coregonus pollan*, pollan | 25 |
| *Coregonus schiuzi* | 26 |
| *Coregonus wartmanni*, Lake Constance whitefish | 16 |
| *Salmo trutta*, brown trout | 6 |
| *Anadromous* | |
| Clupeidae | |
| *Alosa sappidissima*, American shad | 22, 125 |
| Gadidae | |
| *Microgadus tomcod*, Atlantic tomcod | 91 |
| Percichthyidae | |
| *Morone saxatilis*, striped bass | 29, 100 |
| Salmonidae | |
| *Salvelinus alpinus*, Arctic char | 118 |

varied by a factor of 24 among larvae of different sizes. White croaker (*Geryonemus lineatus*), the species with the smallest larvae (1.8 mm at hatch), first fed after 3 d (Watson 1982). In contrast, the largest species, garfish (*Belone belone*), 13.5 mm at hatch, first fed after only 5 h (Rosenthal and Fonds 1973). The longest time to first possible feeding, 5 d, was shared by a variety of small marine, freshwater, and anadromous species.

Hypothesis 2 was also supported. Contrary to expectation, a linear model gave the best fit to the data (Fig. 4). In species for which we have data, times to PNR varied from an average of 3.4 d for the smallest (1.9 mm) lined sole (*Achirus lineatus*) (Houde 1974) to 18 d in the largest (9.4 mm) lake herring (*Coregonus artedii*) (John and Hasler 1956). Differences in temperature between studies could have a large effect on time to PNR. However, when standardized to 15°C to remove temperature effects, the $r^2$ changed only slightly (Fig. 4). This suggests a robust size-dependent relationship independent of temperature effects. Large values of PNR may be underrepresented in the data set, as experiments on a number of larger species were ended after a set time, often before the larvae had reached their PNR. Data from such species could not be included in this analysis.

To evaluate hypothesis 3, we analysed the difference between PNR and first feeding using only those six studies that reported both variables. These restricted data conform to the general regression analysis for the first two hypotheses found in the entire data set (Fig. 4). Regression analysis indicated that the difference between PNR and first feeding increased signifi-

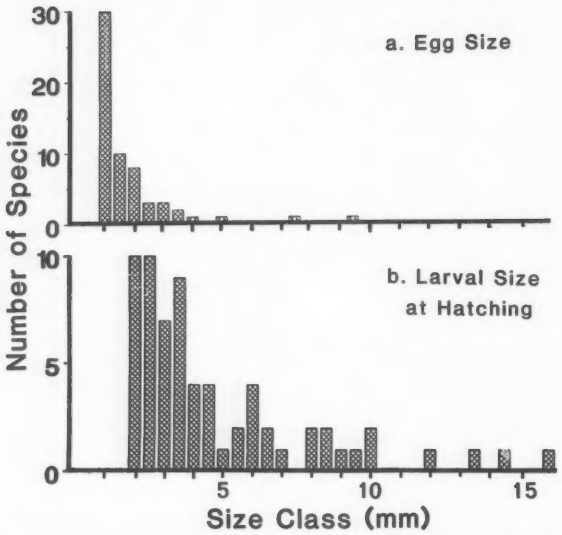


FIG. 1. (a) Frequency distribution of egg diameter of 40 species of freshwater and marine fishes included in this analysis. (b) Frequency distribution of larval total length at hatching for 66 species of freshwater and marine fishes included in this analysis. In both cases, some species are represented more than once.

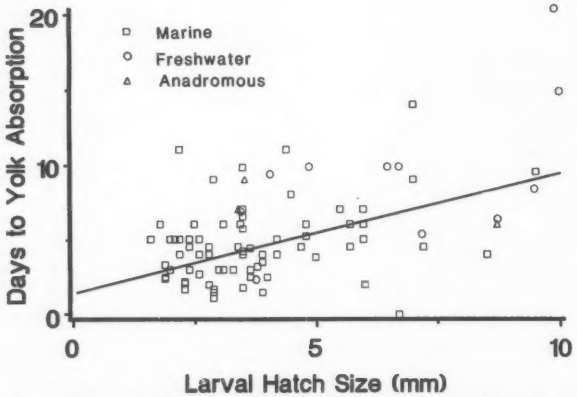


FIG. 2. Time to yolk absorption as a function of larval total length at hatching. Days to yolk absorption = 2.06 + 0.782 length, $r^2 = 0.26$, $p < 0.0001$, $n = 88$ independent estimates of time to yolk absorption for 58 species. When standardized to 15°C: days to yolk absorption = 4.76 + 1.3 larval length, $r^2 = 0.33$, $p < 0.0001$, $n = 88$.

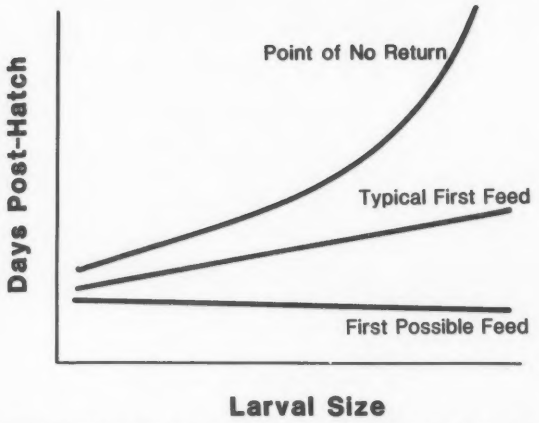


FIG. 3. Hypothetical relationship between different points in the feeding chronology of larval fish and larval length at hatching.

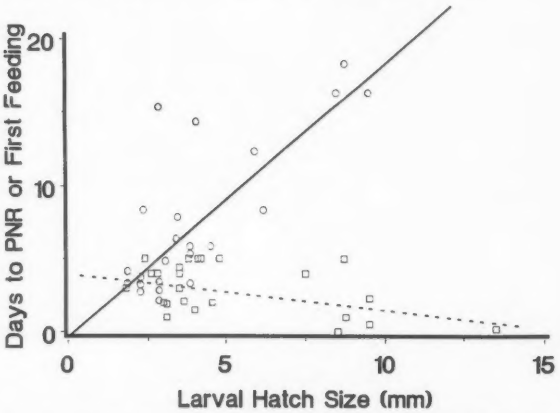


FIG. 4. Time to point of no return (circles) or to first possible feeding (squares) as a function of larval total length at hatching. Using all available data, the regression equations are as follows: days to first possible feeding = 4.09 − 0.237 length, $r^2 = 0.169$, $p = 0.04$, $n = 25$ independent estimates based on 19 species (broken line); days to PNR = 1.82 length − 0.19, $r^2 = 0.61$, $p < 0.0001$, $n = 22$ independent estimates based on 16 species (solid line). When standardized to 15°C: time to PNR = 2.29 + 2.09 length, $r^2 = 0.57$, $p < 0.0001$, $n = 25$. Regression equations were similar for six species for which both time to first possible feeding and PNR were measured: days to first possible feeding = 5.39 − 0.55 length, $r^2 = 0.52$, $p = 0.10$; days to PNR = 0.58 + 1.90 length, $r^2 = 0.69$, $p = 0.38$.

cantly with larval size ($y = 2.46$ length $- 4.809$, $r^2 = 0.89$, $p < 0.0044$, $n = 6$). Even small increases in body size at hatching apparently confer large benefits in terms of flexibility in time to first feeding. For every 0.1-mm increase in length at hatching, larvae gain about 6 h in which to find food; for each 1-mm increase in length at hatching, the "window of opportunity" to feed opens nearly 2.5 d. Larvae that hatch at a large size have a broad "window of opportunity" between the time to first possible feeding and the time to PNR during which they can find appropriate food.

Based on hypothesis 3, we hypothesized that the time when larvae first typically feed should increase and become more variable with increased hatching size (Fig. 3). The determination of time to first typical feeding in the literature is less precise than that for first possible feeding. Accordingly, our interpretation is more cautious. Measures of first typical feeding may depend as much upon when food was offered as upon when the fish were first able to feed. Nevertheless, this measure lends support to our hypothesis. We found a significant increase in typical times to first feeding with increased hatching size (time to first typical feeding = 2.14 + 0.678 length, $r^2 = 0.178$, $p = 0.0006$, $n = 63$). More interestingly, species with large sizes at hatching also showed greater variance in timing of first feeding. To illustrate this point, the absolute value of the residuals of the predicted values in the time to first feeding regression increases significantly with hatching size ($r^2 = 0.658$, $p$

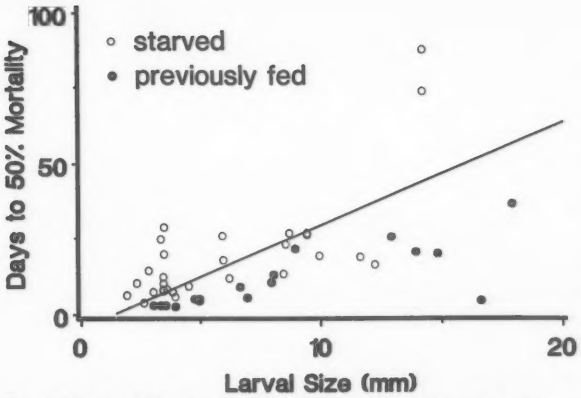


FIG. 5. Days to 50% mortality for a cohort of starved larvae as a function of larval total length at hatching. Days to 50% mortality = 3.60 length − 4.36, $r^2 = 0.52$, $p < 0.0001$, $n = 29$ independent estimates based on 21 species. When standardized to 15°C: time to 50% mortality = 1.27 + 4.17 length, $r^2 = 0.58$, $p < 0.0001$, $n = 29$.

$< 0.0001$, $n = 63$). This reinforces the expected increase in "window of opportunity" to first feeding with increases in larval size at hatching.

While time to PNR is a meaningful measure of larval ability to survive starvation, it is difficult to establish empirically. Instead of PNR, many investigators have measured response to starvation as the time to 50% mortality for a batch of larvae. These data showed the same trend as time to PNR; time to 50% mortality increased significantly with larval size (Fig. 5). Again, temperature may have a strong effect on time to 50% mortality. When we standardized the data to 15°C to account for these effects, a slightly improved fit was obtained. The standardized equation accounted for an additional 6% of the variation in the data. This suggests a robust size-dependent relationship, independent of temperature.

We have been emphasizing the importance of the first feeding in larvae because it is generally believed that once larvae have fed, the risk of death due to starvation is substantially reduced. This is the kernel of Lasker's (1975, 1978) interpretation of Hjort's (1914) critical period hypothesis. To assess this, we compared resistance to starvation in larvae that had already started to feed with those that had never fed. There was a significant interaction between prior feeding and length on time to 50% mortality ($F_{1,42} = 6.5$, $p = 0.015$). Regardless of species, at any given length, we found equal or greater times to 50% mortality in those larvae that were relying on yolk reserves when compared with those that had fed and were subsequently starved (Fig. 5). This suggests that first-feeding larvae are no more vulnerable to starvation than larvae that are of a similar size which have previously fed and subsequently starve. Larvae appear more susceptible to starvation at first feeding only because that is when they are smallest! The influence of starvation or nutritional state on the performance of larval fish should be considered for larvae beyond the first feeding stage of their life history.

In summary, we suggest that a larger hatching size in fish confers resistance to starvation as a result of an increased time to 50% mortality, increased time to PNR, and increased flexibility in the timing of first feeding.

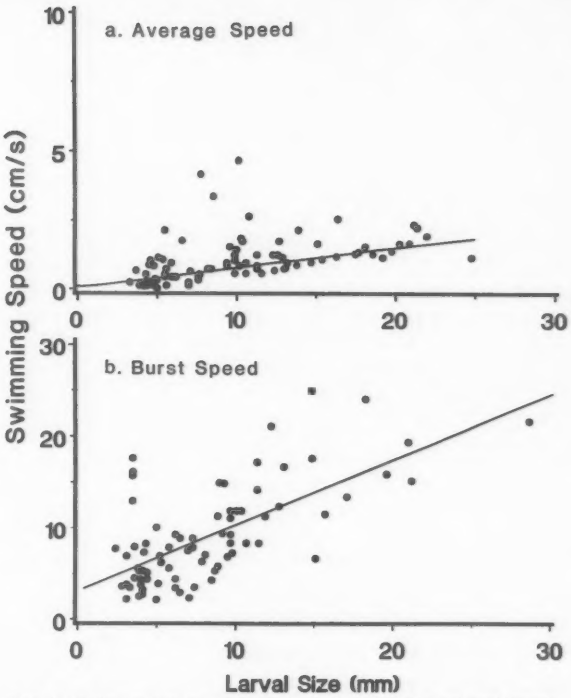


FIG. 6. (a) Average swimming speed as a function of larval total length. $\log_{10}$ speed = 1.07 $\log_{10}$ length − 1.11, $r^2 = 0.46$, $p < 0.0001$, $n = 93$ independent speed measurements on different sizes of larvae of nine species derived from 17 studies. (b) Burst swimming speed as a function of larval total length. Burst speed = 3.33 + 0.71 length, $r^2 = 0.45$, $p < 0.0001$, $n = 76$ independent speed measurements on different sizes of nine species derived from 17 studies.

## Activity and Larval Size

Two potential determinants of larval survival are encounter rate with food (Laurence 1972) and ability to escape predators (Webb 1981). Both are influenced by larval activity. Consequently, the pattern and extent of larval activity may be an important factor in determining growth and survival of larvae (Webb and Corolla 1981; Weihs 1980).

Large larvae generally are more developed at hatching and have more energy reserves than small larvae. We therefore hypothesized that large larvae would become active earlier than small larvae. The data on larval activity are extremely limited but suggest that larvae that hatch at small sizes do have a longer time to maximum activity than do large larvae. Northern anchovies (2.9 mm) do not reach maximum activity until they are more than 4 d post-hatch (Hunter 1972); bloaters (9.5 mm) actively swim 100% of the time within 6 h of hatching (Rice et al. 1987a). Several other examples of small larvae generally agree with the anchovy data (Blaxter 1986), but we need additional data on the activity of species of intermediate and larger sizes to further assess the exact form of this relationship.

Larvae characteristically exhibit two types of swimming (Webb and Weihs 1986). Sustained swimming at almost constant speed is most common and is used primarily in searching for food (Hunter 1972). Larvae also swim in fast bursts which serve as both attack and escape responses and have a high energy requirement (Webb and Corolla 1981; Eaton and DiDomenico 1986). The size dependency of both swimming modes has long

been recognized (Bainbridge 1958: Webb 1975; Blaxter 1986). Following Blaxter (1969, 1986) and Hunter (1972, 1981), we proposed that both within and among species, sustained and burst speed would be accelerating positive functions of larval length.

In agreement with this hypothesis, sustained swimming speed was positively related to larval length (Fig. 6a). Burst swimming speed also increased with larval size (Fig. 6b), affording large larvae an advantage in the ability to gain food or escape predators. The swimming speed data we reviewed were variable. Differences in the swimming ability of species with substantially different morphologies at the same body size account for some of the observed variation (Webb and Weihs 1986). The differing times over which performance was measured may also account for some of the variation. In addition, a variety of methods have been used to estimate swimming speeds, such that the same species can be ascribed quite different abilities (e.g. herring, *Clupea harengus*) (Rosenthal and Hempel 1969; Blaxter and Staines 1971).

In summary, swimming ability is correlated with body size in larval fishes. Large larvae probably become active earlier than small larvae, affording them increased mobility. Moreover, not only can large larvae swim for longer periods of time, they also can swim faster. This greater speed is apparent both in sustained and burst performance. This implies that large larvae experience a higher encounter rate with food. Additionally, they will experience a higher encounter rate with potential predators (see de Lafontaine and Leggett 1988), but are likely better able to escape them once encountered.

## Vision and Larval Size

Vision is an important sense in larval fish, influencing feeding ability and predator avoidance (Blaxter 1986). Changes in the complexity of the eye during development lead to increased visual performance as measured by reactive distance (Blaxter and Staines 1971), visual acuity (Rahmann et al. 1979; Neave 1984), and sensitivity to light (Blaxter 1968). At hatching, the eyes in many teleosts are nonpigmented and not fully functional. Consequently, the rate at which visual development occurs may well regulate survival by influencing when feeding begins. We hypothesized that reactive distance should increase with larval size because visual performance is related to larval development. Search volume, the product of reactive distance and swimming speed, should exhibit a similar increase with larval size.

The limited data available are completely summarized in Blaxter (1986) and support these expectations. The relationship between reactive distance and size is much stronger within a species in a particular study than across species. This may be due in part to investigators using different definitions of reactive distance. In some studies, the estimate of reactive distance used may more accurately be interpreted as a strike distance, and consequently, the distances may not be comparable from one study to another (e.g. Rosenthal and Hempel 1969; Blaxter and Staines 1971). Further, prey sizes were different between studies, also contributing to variation in the estimates of reactive distance (O'Brien 1979). In general, however, reactive distance increases significantly with larval length ($\log_{10}$ reactive distance $= 1.56 \log_{10}$ length $- 1.92$, $r^2 = 0.58$, $p < 0.0001$, $n = 31$ independent estimates based on three species derived from four studies).

Published data on search volume are also completely summarized in Blaxter (1986). Search volume also increased significantly with larval length (search volume $= 0.307$ length $- 1.237$, $r^2 = 0.421$, $p < 0.0001$, $n = 51$ independent estimates based on seven species derived from eight studies). The limited range of reactive distances reported suggests that size-dependent swim speeds probably have a significant influence on search volume estimates (Blaxter 1986). In addition, laboratory studies may have underestimated swim speeds by up to 20% due to sensory deprivation (Westernhagen and Rosenthal 1979), which may increase the variability in the data. Nonetheless, general trends in the vision data among species are similar (Blaxter 1986).

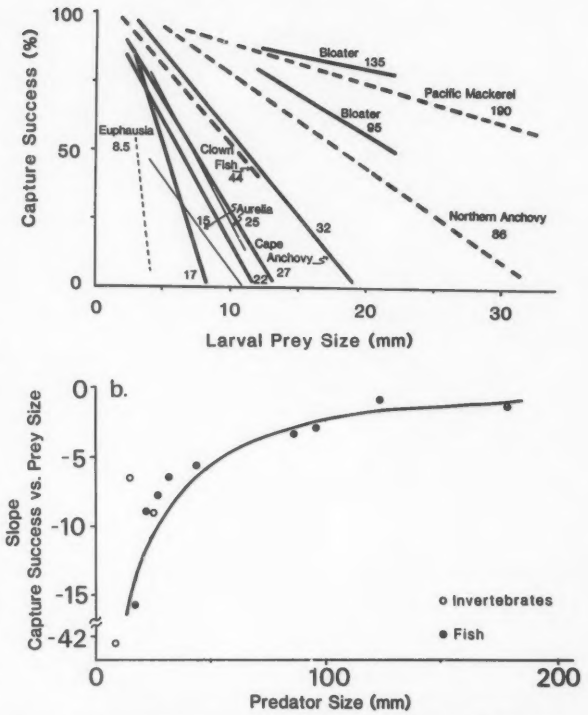


FIG. 7 (a) Capture success for different species and sizes of predators on different species and sizes of larval prey. Linear regressions were fit to the original data so that slopes could be compared. Predator species and sizes are listed adjacent to each line. Heavy lines indicate vertebrate predators; thin lines indicate invertebrate predators. Heavy solid lines for bloater and cape anchovy indicate cannibalism, light solid lines indicate larval herring as prey, and broken lines used northern anchovy as prey. All studies were done in relatively small aquaria with no alternative prey available. (b) Slopes of % capture success versus prey size regressions from Fig. 7a as a function of predator total length. Fish predators are indicated by solid circles; invertebrate predators are indicated by open circles. A large negative slope indicates a rapid decline in capture success with increasing prey size. The fitted equation is slope $= -((\text{predator size} + 63.8)/255)^{-2.53}$.

## Predation and Larval Size

Predation has frequently been cited as a significant cause of mortality in both fish eggs and larvae (Crowder 1980; Hunter 1981). The predators may come from a variety of taxa including

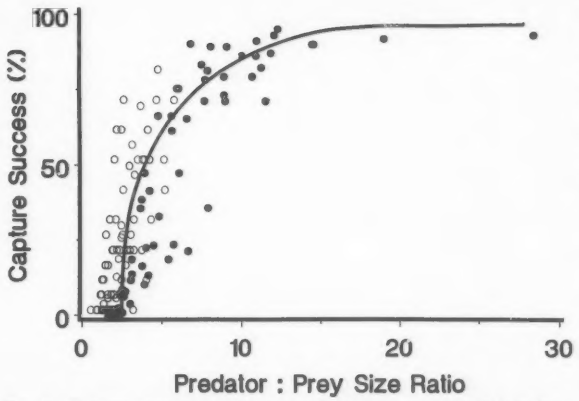


FIG. 8. Capture success as a function of predator to prey length ratio. Points are original data from regressions included in Fig. 7a. Open circles represent invertebrate predators and solid circles represent fish predators. The equation for the fitted line is capture = 100 − ((ratio + 3.37)/44.76)$^{-2.28}$.

several phyla of marine invertebrates, other fishes, and even conspecifics (Hunter 1981; Brownell 1985). Although many factors influence the impact of predation, sizes of both predators and prey have been found to be important determinants of predation in aquatic systems (Brooks and Dodson 1965; Werner and Gilliam 1984; Kerfoot and Sih 1987). The ratio of predator size to prey size has been suggested as a reasonable scalar of predator–prey interactions (Werner and Hall 1974; Werner 1977; Werner and Gilliam 1984; Crowder 1985).

In general, we expected that probability of capture per attempt would decline with increasing larval length (Folkvord and Hunter 1986). When alternative prey are available, one might expect risk of predation by a predator of a particular size to depend upon the relative sizes of prey (Werner 1977; Werner and Gilliam 1984). Both small and large prey may be of lower utility (sensu Werner 1977) than those of intermediate sizes. In terms of optimal foraging theory, risk of predation for larval fish of a particular size should depend upon the size structure of the predator field and the abundance and utility of alternative prey. Unfortunately, few experiments on predation risk for larval fish have been performed with alternative prey (but see Pepin et al. 1987; de Lafontaine and Leggett 1988).

Published experimental studies on size-based patterns of predation on larval fish included predation by both invertebrates and other fish species, as well as cannibalism. We used capture success, the percentage of capture attempts observed that were successful, as a measure of size-based predation risk because few studies estimated predation rates or functional responses to prey density directly.

We investigated capture success of different sized predators as a function of prey size by fitting simple linear regressions to the published data for each predator–prey pair (Fig. 7a). Two features of the predator–prey interactions are evident. First, small predators are restricted to both smaller sizes and a narrower range of prey than large predators. Second, the capture success of small predators is influenced more dramatically by changes in prey size than is success of large predators. To demonstrate this, we plotted the slopes of the capture success regressions as a function of predator size (Fig. 7b). These slopes appear to describe one functional relationship despite the fact that they include different types and sizes of predators from euphausiids (8.5 mm) to adult Pacific mackerel (*Scomber japonicus*) (20 times larger). A steep slope indicates a large change in capture success for a small change in prey size. As a predator's size increases, its expected capture success will be influenced less by prey size. For example, predators greater than 120 mm are affected little by the size of larval prey once encountered, yet for predators less than 50 mm the size of prey attacked is critically important in determining capture success. However, even though 190-mm Pacific mackerel have a high probability of capture for 10-mm northern anchovy larvae, they may not select these prey in the field if alternative prey are available.

To place these relationships in a more general context, we analyzed the relationship between capture success and the predator–prey size ratio using the raw data rather than the slopes of the relationships from individual studies. The results further underscored the importance of size in regulating predation processes as well as the generality of this dependence (Fig. 8). Capture success varied from zero in several cases to a maximum of 96% for 135-mm bloater eating 10.9-mm bloater larvae (Rice et al. 1987a). There was no substantial difference between the capture successes of similar sized vertebrate and invertebrate predators which included body forms as different as euphausiids and jellyfish. The analysis suggests that a predator 15–17 times larger than its prey would experience 100% success per attempt at capturing prey. Below this size ratio the relative sizes of predator and prey exert considerable influence on capture success. In fact, the smaller the size ratio, the greater the influence of changes in size in determining capture success.

In summary, the incidence of predation is affected by both predator size and prey size. Larvae may drastically reduce their susceptibility to smaller predators by outgrowing them. But there seems to be no larval size refuge from predators that are 15 or more times the size of the larval prey. Future studies should examine risk of predation with alternative prey, so that prey selectivity for larvae can be assessed in more realistic terms.

## Discussion

Size-based scaling of physiological and ecological processes is well known from juvenile and adult fish (Brett and Groves 1979; Mittelbach 1981; Werner and Gilliam 1984) and has been broadly applied to other ecological questions (Peters 1983; Calder 1984). Until now, no analyses had been done for larval fish. We recognized that species of larval fish differ dramatically in morphology, developmental status at hatching, environmental temperature, and physiological or behavioral capabilities, so we questioned whether body size alone could integrate the existing data. Although body size alone clearly has limitations, we were pleased by how well the published data fit our general size-based expectations.

In particular, we view several of the results of this analysis as contributing new insights. Most important was the commonality of the relationships we found among fishes from diverse taxa and different habitats. Clearly, the importance of starvation as a factor in the recruitment of larval fish diminishes with increasing size. Larvae that are large at hatching are resistant to starvation, but more importantly, they have a greater flexibility (window of opportunity) in first feeding times. For example, herring from different geographic stocks hatch out at body sizes between 6 and 8.2 mm (Blaxter and Hempel 1963). If we ignore temperature, our analysis suggests that at this dif-

ference in body size, the larger larvae would have about 5.5 d more time to find appropriate food before they reach a point of no return. This suggests that even small size differences of larvae at hatching can have significant ecological implications and that even within a species (e.g. herring), size at hatching might be an adaptive response to local geographic conditions.

We found no evidence to suggest that first-feeding larvae are any more vulnerable to starvation than larvae of a similar size which have been fed and subsequently starved (if anything, the reverse is true because the first-feeding larvae often still have yolk). This suggests that nutritional condition may be important for larvae at any stage; there is nothing "critical" about the first feeding period per se. Small larvae are simply more vulnerable to starvation than larger larvae. Not surprisingly, studies promoting the starvation hypothesis and a "critical period" at first feeding (Lasker 1975, 1978, 1981) involved relatively small marine larvae. If this work had been done with a larger larva, perhaps the current emphasis on the starvation hypothesis in the literature would not have been so strong.

The strong size dependence of capture success shown among predators from euphausiids to adult Pacific mackerel has substantial implications for understanding predation mortality of larvae. It suggests that if the size of a larva is known, some predators can be eliminated a priori as being too small to have much effect, while others which may be important can be identified from the extent of spatial overlap with appropriately sized predators. Surprisingly, the predators on larval fish need not be large or have a typical predator morphology. Most predators in our review could capture prey up to 40–50% of their body length. Probability of capture per attempt approaches 1.0 as the predator–prey length ratio approaches 15–17.

Further generalization regarding predator–prey interactions will become possible if two limitations can be addressed. First, the existing data are for probability of capture per attempt; there is little basis to model or predict the encounter rate between predators and larvae. Second, we know little about prey choice by predators once prey (larval fish) are encountered. The available experiments on predation on fish larvae generally were done without alternative prey (but see Pepin et al. 1987; de Lafontaine and Leggett, 1988). If we are to understand the potential effects of predators on larval fish recruitment, we need to be able to forecast prey choice in predators (i.e. will they select larval fish from among their alternatives?). We will also need estimates of consumption rates (e.g. functional responses of predators to larval fish density with and without alternative prey). Some of the current optimal foraging models (Werner 1977; Mittelbach 1981; Werner and Gilliam 1984) and bioenergetics models (Kitchell et al. 1977; Kitchell 1983) should prove helpful as starting points.

It is important to note that starvation and predation cannot be considered to be alternative (or independent) recruitment mechanisms. Because predation risk is size dependent, growth rates will directly influence the probability of mortality due to predation (Post and Prankevicius 1987). Starvation or poor nutrition can directly influence risk of predation by reducing swim speed or escape ability of larvae (Frank and Leggett 1982; Rice et al. 1987a). Reduced growth rates also prolong the period of vulnerability to gape-limited predators (Werner and Gilliam 1984), leading to reduced recruitment. Clearly, we need to better understand how feeding, growth rates, and risk of predation interact in the ecology of larval fishes.

Some of the relationships we demonstrate are neither new nor unexpected. Several previous workers have published similar results (e.g. Blaxter 1986). Previously, however, the effect of size has not been comprehensively considered. It should be clear that many of the traits examined interact. An advantage in one leads to an advantage in another, forming a positive feedback loop. Consequently, even tiny initial size differences may translate into substantial differences in survival and subsequent recruitment (Adams and DeAngelis 1987). In light of this, we feel that it is important to focus research efforts on individuals rather than populations. Population statistics such as average growth rate or cohort mortality rates may contain less information than an analysis based on individual larvae (Rice et al. 1987b). As the average larval fish in a cohort dies in the first week of life, it is more relevant to know what is unique about the survivors.

Recently, several techniques have been developed that allow us to follow individuals. Otolith analysis, where possible, allows characterization of individuals. In this way, patterns of survival can be related to abiotic and biotic variability (Methot 1983; Crecco and Savoy 1985; Rice et al. 1987b). There is evidence that scale ageing could be used in a similar way (Healey 1982). The increased power of modern computers allows for growth and survival models that follow the fates of individuals. Huston et al. (1988) clearly demonstrated the advantages gained by using individual-based analyses. Such an analysis allows abiotic factors such as temperature and biotic factors such as food availability and predation to be integrated to forecast individual growth rates and survival (Kitchell et al 1977; Stewart and Binkowski 1986). Finally, new genetic techniques, such as those involving mitochondrial DNA, may afford a finer understanding of the pattern of reproductive success of individuals in a population (Wilson et al. 1987).

The approach we present here lends itself to addressing individual-based questions. It suggests strongly that even subtle differences in size between individuals may have profound effects on their fates. Not only is an average value important, but the variance around that average may have equal importance. Further, our approach implicitly recognizes the importance of interactions between factors controlling survival in larval fish.

The general size-based framework we have proposed is not meant to be the final statement, nor is it meant to be predictive for particular species. But it does seem useful as a general context in which to think further about the early life histories of fishes. If larval size at hatching is known, the relationships we report provide important insights into which factors are likely to be important to the survival and growth of individual larvae. But more importantly, there may be factors which can be disregarded a priori. Further, small differences in larval length at hatching can have profound implications for which recruitment mechanisms are likely to be important. This framework has already begun to assist us in addressing old conflicts in the literature; we hope it also admits new questions that more readily take advantage of the previous research on recruitment mechanisms in larval fishes.

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## Appendix

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